INTERNATIONAL SYMPOSIUM ON PERTUSSIS

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INTERNATIONAL SYMPOSIUM ON PERTUSSIS

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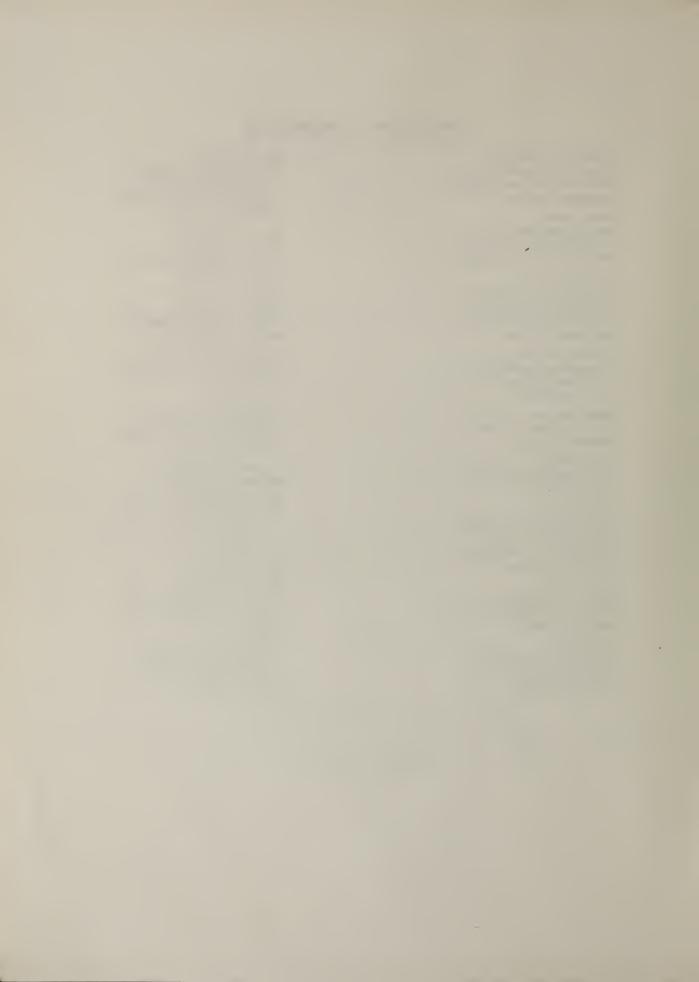Food and Drug Administration
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PREFACE

Throughout the world scientists and public health administrators are examining the role of pertussis vaccine in the control of pertussis. This has provided an impetus to evolve the basic knowledge necessary for the development of an improved pertussis vaccine.

Present pertussis vaccines are, for the most part, crude whole cell preparations that have not benefited from the application of modern immunology, biochemistry, and genetics. We are obliged to accept a "reasonable amount of toxicity" in order to achieve a "reasonable amount of potency." Little or nothing is known of the host-parasite interrelationships in pertussis, and even less is known of the immunochemistry of *Bordetella pertussis*. Field trials to evaluate a new vaccine candidate will be fraught with moral, ethical, technical, and logistic problems and may require many years to complete. Therefore, a broad attack to obtain knowledge on all aspects of the genetics and physiology of *B. pertussis*, the immunochemistry of pertussis vaccine, and the host-parasite relationships in pertussis infection/disease will be necessary to assure the best chances for success in developing the definitive, safe, and effective immunogen.

In 1962, the Permanent Section of Microbiological Standardization of the International Association of Microbiological Societies organized the first Symposium on Pertussis Immunization in Prague. Seven years later, in 1969, the second International Symposium on Pertussis was held in Bilthoven. The third International Symposium on Pertussis in Bethesda in 1978 was planned with the following objectives.

> To hold a scientific meeting committed to the exchange of knowledge and the encouragement of international cooperation in studies to understand the host-parasite relationships in pertussis and to solve problems associated with the production, control testing, and administration of pertussis vaccine.

> To generate a definitive publication which addresses the following:
> —review, acknowledge, and document all previous pertussis research,
> —report current research,
> —develop a consensus of the current status of knowledge concerning host-parasite relationships in pertussis and the use of pertussis vaccine in the control of pertussis,
> —determine if problems or gaps exist in our knowledge and define them, and
> —delineate research areas and goals to be pursued.

Free and open discussion and exchange of ideas characterized the scientific sessions. Probably the most encouraging result of the Symposium was the participation of large numbers of scientists, particularly those new to pertussis research.

Those who were invited to present position papers were limited in their oral presentations by the constraints of time but were encouraged to submit manuscripts which contained complete reviews and bibliographies of the assigned topics. Similarly, authors of individual research papers were provided sufficient space so that the details of their materials, methods, and results could be documented. The authors have responded admirably and the collective manuscripts should serve as an important source document for those engaged in pertussis research and public health administration.

The current debate over the use of pertussis vaccine and the awareness of the need for basic research on *Bordetella pertussis*, pertussis, and pertussis vaccine dictated that the proceedings of the Symposium be published quickly in order to assist scientists in their work and public health

administrators in their policy determinations. Manuscripts were submitted prior to the Symposium and, when necessary, discussed with the respective authors during the meetings. In order to save time, however, the papers were published without returning the edited versions or galley proofs to the authors for review. Accordingly, the editors accept the responsibility for inadvertent omissions or errors incurred during editing and printing.

Charles R. Manclark
James C. Hill
Bethesda, Maryland
November 1978

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* Person who presented paper

Opening Remarks

L. Jacobs

This symposium is sponsored by a number of organizations and interests. We find, actually, that practically every symposium that is held in the NIH now involves a whole variety of institutes. The subjects transcend the interests of a single institute or division, or even of agencies such as the Center for Disease Control, or the Bureau of Biologics of FDA, and so on.

The arrangement is that the Fogarty International Center will help in the organization of these meetings and will do a great deal of the logistics work associated with them. I have the pleasant duty of starting these meetings and welcoming you. They are occurring so frequently that I am having a little trouble finding deep, philosophical thoughts to express at each one. But at this particular one I do not have much trouble at all, because I have had past involvement in some of the problems associated with the subject of this meeting.

At a meeting yesterday regarding the program of the World Health Organization, somebody made the statement that in medicine, technology does not continually progress as it does in other fields such as engineering. A comparison was made to the development of brakes on automobiles and continuing to try to make the brakes more effective and less expensive. The allegation was made that when a modality of treatment or prevention is found in medicine, people move on; they lose interest in it, and they are satisfied that they have something and go on to other things.

I cannot think of a better example to negate that allegation than the work that has gone on in pertussis continually over many years. Everybody has realized that there were some successes with the pertussis vaccine, but they were always perplexed with the difficulties that occurred with it occasionally. Dedicated workers have been operating in the field for a long time, trying to discern the causes of the difficulties with the vaccine and trying to make it more effective.

In these days, when immunization policy has soared to high political levels—indeed, so high that Secretary Califano was invited to talk at this meeting and would have come, had he had time—the question of immunization policy and the efficacy and safety of individual vaccines is a very important one. I am sure that the papers that are presented and discussed at this symposium will be very important in regard to the future of pertussis vaccination.

Opening Remarks

W. Hennessen

It was a great honor to be asked to deliver some opening remarks for our symposium on pertussis. As the President of the International Association of Biological Standardization, one of the sponsoring organizations, I am very pleased that after our two previous meetings on pertussis—the first in Prague, and the second in Bilthoven—our third conference has attracted so many scientists from all over the world.

Since the discovery of the etiologic agent of pertussis by Bordet and Gengou, eight decades have passed, and the attempts to vaccinate go into their fourth decade. But there are still many problems to be solved, both in pathogenesis and immunology.

Judging from the intensity of the planning by the organizers and knowing the expertise of all the contributors, I am certain that this conference will be a step in the right direction. This symposium has been conceived to provide a platform for the exchange of controversial attitudes and opinions. At the end of our meeting, if some of the gaps of controversy can be bridged by new approaches, this meeting will have been a success.

Part 1. PERTUSSIS INFECTION AND DISEASE

Chairman: *Povl Elo Christensen*
Rapporteur: *Vagn Andersen*

Host-Parasite Interactions in Pertussis

C. C. Linnemann, Jr.

INTRODUCTION

As with many common infections of man, the host-parasite interactions in *Bordetella pertussis* infections are incompletely understood. The accumulated knowledge of *B. pertussis* infections, in all its complexity, still represents only pieces of a puzzle, complete only in the major outlines. The key pieces of the puzzle are still unknown.

In any infection, the parasite interacts with the individual host and the community or collective host. At the level of the individual, *B. pertussis* can be traced to the point of attachment in the respiratory tract, then picked up again in the clinical manifestations of the disease. But the chain of events in pathogenesis between these two stages is obscured in a variety of cellular components and properties whose disease-producing roles are not defined. At the level of the community, changing patterns of disease can be traced over recent decades, but the interpretation of these changes is subject to debate. The relationship of host and parasite becomes even more complex if the interaction of other infectious agents in the same host is considered, such as *B. pertussis* and adenoviruses in children with the pertussis syndrome.

In this review of selected aspects of the host-parasite interaction in pertussis, the existing knowledge of *B. pertussis* infections is presented as a background to emphasize the remaining questions.

HISTORICAL BACKGROUND

"Infantum pertussis, quam nostrates vocant *Hooping* Cough," Sydenham, 1676 (1).

Whooping cough, the most characteristic clinical presentation of *B. pertussis* infection, has been recognized as a distinct entity at least since the 16th century. An account by Guillaume de Baillou or Ballonius (1538–1616), published in 1640 (2), is generally accepted as the first clear description of epidemic whooping cough:

> At the close of summer almost the same diseases prevailed as before. . . . Principally that common cough. . . . Serious are the symptoms of this.

> The lung is so irritated by every attempt to expell that which is causing the trouble it neither admits the air nor again easily expells it. The patient is seen to swell up and as if strangled holds his breath tightly in the middle of his throat. . . . For they are without the troublesome coughing for the space of four or five hours at a time, then this paroxysm of coughing returns, now so severe that blood is expelled with force through the nose and through the mouth. Most frequently an upset stomach follows. . . . For we have seen so many coughing in such a manner, in whom after a vain attempt semi-putrid matter in an incredible quantity was ejected.

Since earlier descriptions cannot be identified, some reviewers have suggested that pertussis appeared de novo, a new infection of man, at this time (3). Other historians have suggested that the disease was only new to Europe in the Middle Ages, having been imported from other parts of the world (4). Still others have suggested that although it was a common problem, pertussis was not treated by physicians, and thus not mentioned in the medical literature of the times (5). The apparent reason for the disagreement among scholars is that historical descriptions of illnesses characterized predominantly by coughing are not sufficiently detailed to allow either the inclusion or exclusion of pertussis. Even Baillou's account does not contain a description of the inspiratory whoop which is the hallmark of the disease. Sticker has concluded that the historical "trail of whooping cough is associated with mental delusions," and one must accept that the exact origins of pertussis cannot be determined (6).

Whooping cough has been known in many countries by many names, including chincough or kink-cough in English, coqueluche in French, kinkhoest in Dutch, keuchusten in German, kighoste in Danish, or kikhosta in Swedish (7). In English, "hooping cough" was generally used until the end of the 19th century, but in the 20th century "pertussis" has gradually replaced "whooping cough" in the medical literature. The original use of "per-

tussis" to denote a serious cough is attributed to Sydenham in the 17th century, and the term is found in the dictionaries of the 18th century (1). This change in terminology may have begun with the use of the term in naming the causative agent (*Hemophilus pertussis*, or *Bordetella pertussis*). As the varied clinical spectrum of *Bordetella* infections has been appreciated, including the fact that the "whoop" may not occur in many patients, the term "pertussis" has come into general use in English-speaking countries for disease caused by *B. pertussis*. It has also been used in an expanded sense to refer to the pertussis syndrome—paroxysmal coughing with an inspiratory whoop and/or lymphocytosis.

THE PARASITE

In 1900, Bordet and Gengou observed a microorganism, which was probably *B. pertussis,* in the sputum of a patient with pertussis; but it was not until 1906 that they first reported the isolation of the organism on a potato-glycerol agar (Bordet-Gengou medium) (8). While examining culture plates with a microscope several days after inoculation, they found the small colonies of *B. pertussis*. The recovery of *B. pertussis* from patients continues to be difficult. The problems with the slow growth of the organism and overgrowth with other organisms have been solved partially by using nasopharyngeal swabs, adding antibiotics to the media, and sealing plates to prevent drying (Linnemann, C. C., and Bass, J. W., in press). With these techniques, a skilled microbiologist can recover *B. pertussis* from the majority of patients cultured early in the illness before antibiotics are given (9). However, most studies report recovery rates of less than 50%, perhaps because the patients are not cultured until late in the illness or after antibiotics have been started, or because specimens must be transported to a qualified laboratory. Few hospitals in the United States have clinical microbiologists experienced in culturing *B. pertussis* or have the special media required for isolation of the organism. Therefore a diagnosis of *B. pertussis* proven by culture is uncommon in many medical centers in this country. But this does not mean that *B. pertussis* is uncommon. Fluorescent antibody staining of nasopharyngeal smears errs in the other direction, producing many false positives in laboratories that do the test infrequently (10).

B. pertussis is one of three distinct species in the genus *Bordetella*, which also includes *B. paraper-*

tussis and *B. bronchiseptica*, despite a recent report of conversion of *B. pertussis* to *B. parapertussis* in the laboratory (11,12). It is a small aerobic Gram-negative coccobacillary organism, 0.2–0.3 μm by 0.5–1.0 μm in size. Pleomorphism develops in older cultures, and the organism undergoes phase variations (phases I to IV), which may be analogous to smooth to rough changes and are associated with a loss of virulence. Capsules have been reported to be present on special staining. Electron microscopy has shown filamentous surface projections that may represent a form of pili or fimbriae and may have a role in attachment (13). Attachment of the organism and the subsequent development of ciliostasis may be important virulence factors. On Bordet-Gengou media, *B. pertussis* produces small, smooth-edged, elevated colonies, which are gray but in reflected light appear silver. The colonies have been said to resemble droplets of mercury or bisected pearls. Depending on the type of blood used in the media, hemolysis may or may not be present.

A variety of biologically active components and properties of *B. pertussis* have been described (Table 1) (14,15). Despite the possibility that these contribute to the pathogenesis of disease in man, there is no direct evidence for such a role. Two toxins have been described: a heat-labile or dermonecrotic toxin and a heat-stable lipopolysaccharide endotoxin. Systemic toxicity is notably absent in pertussis, but a toxin acting locally on the ciliated epithelial cells of the respiratory tract could play a role in pathogenesis. This would be similar to cholera, which is a superficial infection of the gastrointestinal tract mediated by the local effects of a toxin. Agglutinogens, surface antigens which define the serotype, have been implicated as possible protective antigens, but, as will be discussed later, this is unproven. Hemagglutinin is important because it is associated with the pililike surface filaments which may be important in the attachment of the organisms to cells. Finally a lymphocytosis promoting factor (LPF) has been described which apparently produces lymphocytosis by interfering

Table. 1. Biologically Active Components and Properties of *B. pertussis*

Heat labile toxin	Histamine sensitization
Heat stable endotoxin	β-Adrenergic blockade-like effect
Agglutinogens	
Hemagglutinin	Islet cell stimulation
Lymphocytosis promoting factor	Adjuvant effect
	Mitogenicity

with recirculation of lymphocytes (16–18). It seems obvious that LPF is related to lymphocytosis in man, but again there is no direct evidence.

In addition to these components of *B. pertussis,* several other biologic activities have been described, including histamine sensitization, β-adrenergic blockadelike effect, islet cell stimulation, adjuvant effect, mitogenicity, and many others. Recently, Morse has isolated an apparently pure LPF, a protein with a molecular weight of 67,000 to 73,600, which also produces histamine sensitization, refractoriness to epinephrine-induced hyperglycemia, and mitogenicity (19). This suggests that one component of *B. pertussis* is responsible for many of the biologic activities of the organism. "Pertussigen" has been suggested as a term for this component, which is unrelated to the toxins, agglutinogens, or hemagglutinin (15). An extracytoplasmic adenylate cyclase of *B. pertussis* has been described, but its role, if any, in pathogenesis is unknown (20). Despite this long list of biologically active components and functions, the protective antigen or antigens for man have not been specifically identified.

HOST-PARASITE INTERACTIONS IN THE INDIVIDUAL

Pathology

In 1912, Mallory and Hornor described the histological changes in the respiratory tract of children who died from pertussis (21). They observed masses of bacteria between the cilia and extending to the base of the cilia of epithelial cells lining the tracheobronchial tree (Fig. 1). They demonstrated the shedding of infected epithelial cells and noted that in the later stages of disease the cilia were reduced to "stubs" or entirely lost in many areas. There was no penetration of cells by the bacteria or necrosis of the epithelial cells. The cellular response to infection included the migration of polymorphonuclear cells between the epithelial cells and into the lumen, and infiltration of the submucosa with lymphocytes and plasma cells. Organisms were observed in phagocytic cells. Experimentally, the same authors were able to demonstrate similar lesions with attachment of bacteria to ciliated epithelial cells in dogs and rabbits, although Mallory later acknowledged that coincidental *B. bronchiseptica* infections may have occurred in some of his experimental animals (22,23). They suggested that the specific attachment of *B. pertussis* to ciliated epithelial cells had not been appreciated earlier because of the small size of the organisms and the
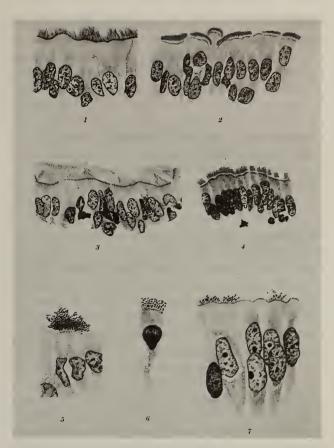
Figure 1. Histological lesion in pertussis. Normal trachea and bronchus (1,3); infected trachea and bronchus (2,4); and late effects, including shedding of a ciliated epithelial cell (5,6 and 7). [From Mallory, F. B.; Hornor, A. A. J. Med. Res. 27:115–123; 1912.]

faint staining with routine stains. They could not decide if the loss of cilia was a direct effect of infection or a postmortem artifact, but they concluded that the basic problem in pertussis was interference with cilia function by the mechanical effect of masses of bacteria.

The attachment to ciliated epithelial cells in the respiratory tract as observed in light microscopy was confirmed by others, and recently Collier and colleagues, using infected hamster tracheal explants, demonstrated by electron microscopy that the bacteria are in close association with cilia, microvilli, and the lumenal border of ciliated epithelial cells (24, 25). There was no attachment to nonciliated cells. After attachment there was decreased protein synthesis, ciliostasis, destruction of subcellular organelles, morphological evidence of injury on light microscopy, and finally expulsion of the cells. The areas from which cells were extruded were filled by nonciliated cells. There was no evidence of interor intracellular invasion. Filtered media from in-

fected cultures did not produce similar changes in uninfected cultures. Matsuyama has also demonstrated the attachment of *B. pertussis* to ciliated epithelial cells in cultures of rabbit tracheal mucous membrane (26). He showed that virulent phase I *B. pertussis* adhered to the site of inoculation in the tracheal culture, but avirulent phase III organisms were moved down the tracheal segment by ciliary action. This difference was observed when both phase I and III organisms were inoculated on the same culture, suggesting that attachment, not ciliostasis, was the critical factor. The mechanism of attachment is unknown. No pili were seen by scanning electron microscopy of tracheal cultures of *B. pertussis* (25). It is interesting that the light and electron microscopy findings in *B. bronchiseptica* infections in dogs are similar to those seen with *B. pertussis,* and fibrillar extensions of the *B. bronchiseptica* were observed radiating toward the cilia (27). Attachment to ciliated cells outside the respiratory tract has been observed in the mouse brain, but Holt and colleagues noted that the attachment was to the microvilli only (28).

The bronchopneumonia which occurs in pertussis may result from *B. pertussis* alone or from secondary infections. Smith described the pathology in culture-proven cases of pertussis as a lymphocytic infiltration of the peribronchial tissues and the alveolar walls (29). This is associated with a mucopurulent exudate in which organisms were seen in the alveoli. Necrosis was absent. The tracheobronchial lymph nodes were hyperplastic with mononuclear infiltration, and bronchiectasis and fibrosis occurred. The exudate may obstruct the bronchioles, producing atelectatic changes, and occasionally, interstitial and subcutaneous emphysema may occur. Other descriptions of the pulmonary changes in pertussis have also noted the presence of edema and hemorrhage, but consolidation only occurs with secondary bacterial infection.

Although the infection appears to remain localized to the respiratory tract, pathologic changes have been described in other organs (30). The literature contains conflicting reports regarding the pathologic lesions in the central nervous system. In particular, the presence of meningitis and encephalitis has been frequently reported but has seldom been supported by a clear description of the microscopic findings or a definite diagnosis of uncomplicated pertussis. Dolgopol clarified this situation by a careful review of the literature and a detailed report of cases which she had examined (31). Cases in which something other than pertussis could have

accounted for the neurologic manifestations were excluded. On gross examination, edema was usually present, and occasionally hemorrhages. The latter could be massive, but were usually small subarachnoid or multiple petechial lesions. The most common microscopic lesions were degenerative changes of the pyramidal cells in the hippocampus and Purkinje cells of the cerebellum, but cells throughout the cortex and other areas of the brain could be involved. These changes were compatible with anoxia. Perivascular hemorrhages were present in both the white and gray matter, with surrounding cellular degeneration. Encephalitis was not seen. She also described lymphocytic "plugs" in many capillaries and veins. Cultures of the brain were negative. She concluded that neither direct infection nor a bacterial toxin was involved in producing these pathological changes. The pathogenesis of the lesions in the central nervous system is not clear, but the changes observed are compatible with anoxia and occasional hemorrhage.

Other pathologic changes in pertussis include hemorrhage into the conjunctiva or retina, fatty infiltration in the liver (not the microvesicular fatty changes described in Reye's syndrome), and lesions resulting from secondary infection (30). It should be noted that it is difficult to interpret many of the pathologic reports in the older literature, because cases were not documented adequately by culture and the changes due to secondary infection were not distinguished from those resulting from pertussis itself. Cases have not been described in detail in the recent literature, because deaths from pertussis occur infrequently.

Clinical Disease

Typical pertussis includes three stages: catarrhal, paroxysmal, and convalescent (30, 32). The catarrhal stage, lasting 1 to 2 weeks, begins with nonspecific respiratory symptoms, malaise, anorexia, and sometimes a low grade fever, and ends with the increasingly severe cough. The paroxysmal stage, lasting several weeks, begins with paroxysmal coughing. During this stage the characteristic "whoop" appears, produced when prolonged coughing is followed by a forced inspiration over a partially closed glottis. The paroxysms of coughing may be followed by vomiting and may be associated with epistaxis, conjunctival or scleral hemorrhages, and periorbital edema. Laboratory findings include leukocytosis and lymphocytosis, which appear late in the catarrhal stage and continue to increase in the paroxysmal stage (33,34). Maximal lymphocytosis

corresponds to the time of most severe coughing. The sedimentation rate is normal or decreased, and some patients may have hypoglycemia (34,35). During the final or convalescent stage sporadic coughing may continue for weeks to months and exacerbations with whooping may occur with other respiratory infections.

This "typical" presentation of the disease represents the most severe form of *B. pertussis* infection. The clinical spectrum of illness also includes asymptomatic infection, symptomatic upper respiratory tract infection, or mild bronchitis. Few studies have defined the relative frequency of these various manifestations in a nonhospitalized population, although it appears that approximately 75% of susceptible children develop clinically recognizable disease as indicated by a previous history of disease (36). Vysoka has reported that more than half of cases have paroxysmal coughing and about a third have nonparoxysmal coughing (37). This is in contrast to *B. parapertussis* infection in which only 20% had paroxysmal coughing. The details of this study were not described. During epidemics of pertussis, there have always been numerous contacts with atypical respiratory symptoms, some of which may represent reinfections. Since the introduction of effective vaccines, there has been a marked decrease in the occurrence of typical disease, in both the total number of cases and the proportion of infected individuals. Madsen noted the decreased severity of pertussis in vaccinated patients during epidemics on the Faroe Islands in the 1920s (38). Although this was not Stewart's experience in Scotland, others have supported Madsen's observations (39,40). In the most recent study, Miller and Fletcher reported that only 2% of vaccinated children 1 to 2 years of age who had pertussis required hospitalization in contrast to 16% of unvaccinated children (41). This decrease in severity of disease in vaccinated children seems to be related to partial immunity.

Host factors other than preexisting immunity affect the clinical manifestations of pertussis. Age is the best defined of these factors. In the young infant both the whoop and the abnormal lymphocytosis may be absent, and the predominant symptoms may be choking or apnea and cyanosis (34,42). The mortality rate is also highest in children under 1 year of age (43). In older children and adults, typical paroxysmal coughing with a whoop may occur, but the illness is usually milder (44). The cough may or may not be paroxysmal, and the whoop and lympyhotocytosis are usually absent.

Other host factors that have been cited as affecting severity of illness include sex, underlying disease, and malnutrition. In contrast to most infections, pertussis is reported to be more common and to have a higher mortality rate in females (43). The reason for this is unknown. Lapin noted that pertussis seemed to be more severe in those with a previous history of allergic disease, but there is little documentation of this observation (30). Reports of high mortality rates still occurring in underdeveloped countries suggest that malnutrition may be a factor in producing severe disease (45).

The prolonged clinical illness produced by *B. pertussis* is associated with prolonged excretion of the organism in the respiratory tract (37). Despite this, the paroxysmal coughing may continue long after cultures become negative. Less than 50% of untreated patients will have positive cultures after the third week of the illness, and 20% or less after the fifth week (46,47). When the patient is treated with an appropriate antibiotic, such as erythromycin, the culture becomes negative within a few days, but, unless treatment was started in the catarrhal stage, the coughing continues unchanged (48). The prolonged cough may be due to the continuing presence of *B. pertussis* in the lower respiratory tract even when routine cultures are negative, but there is no evidence to support this idea. Slow recovery of ciliary function could also contribute to the continuing cough. However, the regeneration time of the ciliated epithelial cells in the human respiratory tract is unknown (49). Animal studies suggest that regeneration requires several weeks, but ciliary function is restored much sooner because of the considerable functional reserve of the mucociliary transport system. Because of these considerations it has been suggested that something more than direct injury of ciliated epithelial cells is involved in the pathogenesis of the prolonged coughing (50). Residual neurologic injury with autonomic dysfunction of β-adrenergic blockade has been postulated as a cause, and on the basis of this hypothesis salbutamol has been used to treat pertussis, with conflicting results (51,52). Steroids have also been used in the treatment and reported to decrease the duration of coughing (53). This is an interesting speculation, but the mechanism of cough production remains unknown.

The most common complications of pertussis are pulmonary, and some are caused by secondary bacterial infection (30). Clinical observations since the introduction of antibiotics have noted the increasing mildness of pertussis with few secondary pneu-

monias and the lack of long-term sequelae (54–57). It is impossible to determine the relative contributions of different factors to the changing clinical disease, but antibiotics may have decreased the frequency and severity of secondary bacterial pneumonias and thus the late sequelae. However, autopsy studies in the preantibiotic era do not clearly identify treatment complications in the majority of patients. Whether antibiotics have contributed to the overall decrease in severity of the disease is unknown. The question could be asked as to whether or not the indiscriminate use of antibiotics for mild respiratory tract infections has prevented the progression to the paroxysmal stage of disease. The fact that pertussis in underdeveloped countries has remained as severe as in the prevaccine and preantibiotic eras, suggests that the virulence of the organism itself has not changed (45).

The most common neurologic complication is convulsion, but paralysis, coma, blindness, deafness, movement disorders, and others have been reported (30, 58). As discussed before, these probably result from anoxia and occasionally from hemorrhage, but other mechanisms have been postulated. Hypoglycemia, which has been observed in pertussis, could account for some of the central nervous system disease, as could the hyponatremia which results from vomiting (35,59). More observations on the biochemical abnormalities in pertussis are needed to determine if these are important factors. As with pulmonary complications, the late sequelae of neurologic complications seem to have decreased, but good data on the exact incidence are not available (56, 58).

Immune Response

Immunity to pertussis appears to be longlasting since the recurrence of typical illness and culture-proven reinfection are rare (60, 61). However, reinfection could be commonplace and yet go unnoticed if only atypical illness occurred. The occurrence of nonspecific cough in adults in contact with children with pertussis ("grandmother's whooping cough" or "nurse's cough") may be such reinfections, but would probably be attributed to viral infections. Huang and colleagues showed that immunized monkeys could be reinfected and could excrete B. pertussis for as long as 18 days without developing symptoms (62). These animals had increasing antibody titers during the reinfection. As with other infections with lifelong immunity, the question arises whether persisting immunity de-

pends on mild reinfection to boost the underlying immunity.

The actual mechanisms of immunity to B. pertussis in the individual host have not been defined, but there have been many studies of specific aspects of the immune response. The initial stage of host defense probably depends on phagocytosis by the polymorphonuclear leukocytes and macrophages. Early descriptions of the pathologic lesions in pertussis referred to phagocytic cells containing organisms (8,21). In murine pertussis, the bacterial population in the lungs increases rapidly after inoculation, then falls abruptly (63). Presumably this is due to phagocytosis, but the organism does not disappear; it remains in the lungs for weeks (64). The final clearance of bacteria requires the development of specific immunity (65).

The humoral immune response to B. pertussis has been studied extensively since 1906, when Bordet and Gengou demonstrated both agglutinating and complement fixing antibodies in convalescent sera from patients with pertussis (8). Serum antibody responses have been measured by a variety of techniques in addition to complement fixation and agglutination, including bactericidal, immunofluorescence, immunodiffusion, passive hemagglutination, and radioimmunoassay tests (66–71). The most notable aspect of these studies is the variability of results. Differences in technique account for many of the discrepancies, as does the lack of sequential observations in individual patients. The basic pattern of the antibody response is illustrated by Donald's 1938 study of complement fixation antibody after infection (66). Antibody began to appear in the second or third week of the illness with the peak at the seventh to eighth week, when 89% of the sera were positive. He noted that the appearance of the antibody corresponded to the disappearance of B. pertussis on culture, which suggests that antibody has a role in recovery from infection. Similar experience has been reported with agglutinating antibody, which may persist for years. The Combined Scottish Study in 1970 confirmed the value of antibody tests in diagnosing pertussis, particularly in older children (72). The agglutination test corresponded well with the complement fixation test. The pattern of the antibody response, with the appearance delayed for several weeks, requires that the convalescent sera be collected late in the illness.

The role of antibody in resistance to infection or reinfection is unproven. Studies in the older literature report the value of convalescent or hyper-

immune serum in preventing disease in contacts, but these observations have not been confirmed in well controlled studies (30). Recent studies have failed to show any significant benefit from hyperimmune globulin in preventing pertussis or treating children in the paroxysmal stage (73,74). Agglutinating antibody levels do correlate with immunity, i.e., the chances of developing pertussis after exposure decrease with increasing antibody levels (75,76). Still, pertussis can develop in the presence of antibody, and resistance can be present in the absence of antibody. This suggests that the serum antibodies being measured do not represent protective antibody but are simply an indirect reflection of the underlying immunity.

One possible explanation for the failure to relate antibody to protection is that serum antibody does not reflect what is happening in the respiratory tract. Studies of the local immune response are limited, but local antibody could be important in preventing adherence of the bacteria to the ciliated epithelial cells and in promoting phagocytosis. Geller and Pittman have reported increased IgG and IgA specific for B. pertussis in the tracheobronchial washings of mice with pertussis by the 15th day of infection (77). Cheers has shown that in murine pertussis the phagocytosis of B. pertussis does not result in complete killing of the organism, but rather a state of equilibrium (63). The infection persists until protective levels of antibody appear, and then the organism is eradicated (65). Holt has reported the development of antibody in the saliva of an infected monkey that interfered with the adhesion of B. pertussis to cells (28). All these animal studies suggest that antibody may be important in recovery from infection. There has not been a study of the local immunity to pertussis in the human respiratory tract, although Thomas did report the presence of antibody by radioimmunoassay in respiratory secretions after vaccination (71).

Cellular immunity in pertussis in man has been studied even less than humoral immunity. The lymphocytosis that occurs in pertussis has been characterized as a proportional increase in both T and B cells, and it is postulated that this results from a failure of lymphocytes to reenter lymph nodes, as suggested by animal studies (78). One retrospective study of children with pertussis who had received BCG reported that they had a less marked response to PPD than would be expected in normal children (79). The authors speculated that the apparent anergy resulted from the inability

of the lymphocytes to migrate to the skin test site. There are numerous and often conflicting studies of cellular immunity in animal models [reviewed by Olson (3)]. The studies of murine pertussis which suggested an important role for antibody failed to show a correlation between the presence of delayed hypersensitivity and the titer of bacteria in the lungs of individual mice (80). However, it was only after the majority of animals developed delayed hypersensitivity to pertussis antigens that B. pertussis disappeared. In evaluations of skin tests for pertussis as a measure of immunity in man, both immediate and delayed hypersensitivity have been reported and may persist for years (30). Morse has reported that the supernatant from B. pertussis cultures contains a nonspecific mitogen for human lymphocytes (14). A specific lymphocyte response to B. pertussis antigens has not been reported. Again, as with the humoral immune response, the role of cellular immunity in recovery from pertussis and resistance to reinfection remains to be determined.

HOST-PARASITE INTERACTIONS IN THE COMMUNITY

Epidemiology

Reservoir and Transmission. The basic epidemiology of pertussis is simple—man is the only known reservoir, and infection is transmitted by symptomatic patients. There are no proven chronic carriers of B. pertussis (81,82). This has been studied extensively by surveys using the current techniques of fluorescent antibody staining and nasopharyngeal cultures using Bordet-Gengou medium containing antibiotics. Subclinical or clinically unrecognized infections do occur, as has been demonstrated in culture surveys of contacts of pertussis patients. In the British studies reported in 1973, 2% of asymptomatic household contacts under 5 years of age were culture-positive, but subsequent followup of these contacts was not reported (83). If followed carefully for the development of symptoms, most asymptomatic culture-positive contacts will become ill, although they may have only nonspecific respiratory tract infection (81). If treated with an antibiotic such as erythromycin, they may remain asymptomatic. Sporadic, culture-proven, asymptomatic infections have been documented, but there is no evidence that these are significant in the transmission of infections. In my experience, this possibility has arisen only once. During an endemic of pertussis in a hospital, the members of one physi-

cian's family developed pertussis. The physician was asymptomatic, but a nasopharyngeal culture was positive, and he was treated with erythromycin. Even though there was no recognized community source for his family's infection, this could not be excluded, nor could the possibility that the physician would have subsequently developed clinical illness if he had not been treated. Still, the possibility remains that the physician had a hospital-acquired, subclinical infection, which he transmitted to his family.

Atypical cases of pertussis may play an important role in the dissemination of disease. In the prevaccine era, an apparent source of infection could be identified in most cases of pertussis. Luttinger in 1918 reported that a source could be determined in 83% of 2,310 cases (84). In more recent times, it has become increasingly difficult to trace the person-to-person transmission in an individual case. Nelson has reported that in very young infants with pertussis the most common source of infection, based on the presence of clinical symptoms, is an adult member of the family (85). We have seen a similar situation in hospitalized children with culture-proven pertussis (Table 2). Equally important is the failure to identify any source of infection in a third of cases. The role of adults in transmission of infection also is indicated by reports of hospital epidemics, where physicians and nurses have transmitted infection to each other and to their patients (86,87). With rare exceptions, these adults have had atypical disease (44). These observations suggest that the reservoir of infection may have changed from the child with typical disease to either the child or the adult with atypical disease. Atypical cases may also have been important in the transmission of disease in the prevaccine era, but were obscured by the large number of typical cases. There is no way to know if the potential for transmission of disease is different for older patients

with symptomatic reinfection or for previously vaccinated patients with atypical primary infections.

B. pertussis appears to be transmitted primarily by droplets. There is no evidence that airborne spread by droplet nuclei occurs or that spread by fomites is important, although Lapin states that the organism has been recovered from dried-out sputum after 3 days (30). The secondary attack rate in susceptible children is extremely high, ranging from 25–50% in schools to 70–100% in susceptible household contacts (88,89). Most secondary cases in the household occur within 2 weeks of the onset of symptoms in the index case (90). These observations suggest that the organism is transmitted rapidly to contacts. An alternative explanation is that this reflects the intense and prolonged exposure that can occur in the catarrhal stage before the index case is recognized as having pertussis. Atypical cases would also provide a source of infection over a long period of time, because these patients continue to expose others throughout their illnesses. After transmission of infection, there is a 7–10 day incubation period. This has been confirmed both experimentally and epidemiologically (91,92). In 1933, two susceptible children were isolated and then infected with *B. pertussis*. Seven days later they developed a cough. Bell has reported eight cases of pertussis in which the exposure occurred on a single day, and in all cases, coughing began 9 days later. Extremes of 5 to 21 days have been reported.

Changing Epidemiologic Patterns. In the prevaccine era the highest age-specific incidence of pertussis occurred in the 4-year-old age group, reflecting transmission of the disease in the preschool population (36). If just the susceptible children in each age group were considered as the population at risk, then the highest incidence occurred in 6-year-old children, reflecting the transmission in schools. By 10 years of age, 73% of children had a clinical history of pertussis. Obviously this has changed since the introduction of vaccine, and it is uncommon in the United States to find any school age child with a history of typical pertussis. There has also been a relative shift in the age distribution of cases to the older age groups. Ipsen and Bowen observed this in Massachusetts in the 1950s after a decade of vaccine usage (93). Although the incidence of pertussis in children under 10 years of age had declined, the incidence remained the same in those over 10 years of age. Similar observations have been made in other countries

Table 2. Sources of *B. pertussis* Infection as Determined by History

Study	No. of Patients	Symptomatic Contacts		
		Children	Adults	Unknown
Cincinnati [a] 1976–77	20	7	6	7
Dallas [b] 1971–77	22	2	12	8

[a] All hospitalized children with culture proven pertussis
[a] Infants < 12 weeks of age, Reference 85

(94). Despite this relative shift in the age distribution, there is no evidence that there has been an absolute increase in the older groups. Recent studies of pertussis outbreaks have reported that a large proportion of cases occurred in older children and adults, but there are few studies from the prevaccine era in which atypical cases were documented in the older age group that can be used for comparison (95). As discussed previously, it is possible that atypical cases were just as common in older age groups before the introduction of vaccine, but were not identified.

Although it has not been proven, there is still a basis for predicting that the incidence of pertussis might increase in older age groups in previously vaccinated populations. Vaccine-induced immunity may not persist as long as immunity induced by natural disease. In 1965, Lambert described an epidemic of pertussis in which he determined the attack rates in previously vaccinated patients by the number of years since immunization (96). The attack rate was only 21% of those who had received pertussis vaccine within the previous 3 years, but was 95% in those who had been immunized 12 years or more before exposure. It is surprising that other investigators have not confirmed this dramatic loss of vaccine-induced immunity with time. This suggests the possibility that persisting immunity in vaccinated populations depends on subclinical or mild infections to booster waning immunity in later years. Lambert's epidemic may have occurred in a population which had not been exposed to *B. pertussis* since vaccination. Studies of hospital employees have also demonstrated higher antibody titers in those with patient contact, and thus presumably exposed to pertussis (86,87). Of course, a similar situation may be involved in maintaining natural immunity, with reinfection enhancing immunity after primary infections.

The most important effect of vaccine on the epidemiology of pertussis has been the decrease in the morbidity and mortality. This is clearly demonstrated by the experience in the United States, where effective vaccines have been in use since the early 1940s (Fig. 2). The incidence of pertussis decreased dramatically at that time, and even though the mortality had been decreasing prior to the vaccine era, the decrease has accelerated significantly since the introduction of vaccine. The relationship between vaccine and the declining morbidity and mortality of pertussis is not as obvious in some countries, such as the United Kingdom,
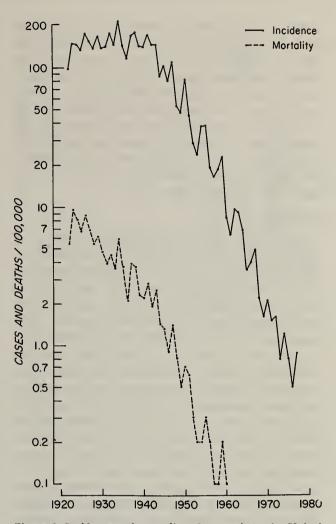
Figure 2. Incidence and mortality of pertussis in the United States. The mortality decreased below 0.1 per 100,000 in 1960. Based on data from the Center for Disease Control and the National Center for Health Statistics, U.S. Department of Health, Education and Welfare.

perhaps as a result of slower introduction and acceptance of pertussis vaccine and problems in providing effective vaccines (97,98). The experience in those countries does indicate the important role of changing socioeconomic factors in the declining morbidity and mortality of the disease (39), but it also supports a causal relationship between vaccination and the decrease in pertussis. The recent experience in England indicates that when there is significant decrease in the utilization of vaccine in a developed country, there is a marked increase in the incidence of disease (99). Conversely, the recent introduction of vaccine into undeveloped countries, where pertussis is still a major health problem, has dramatically reduced morbidity and mortality rates (45).

With the decreasing incidence of pertussis, the possibility of eradication should be considered (100). Investigations in eastern Europe have reported that immunization of 60% of children will contain infection, but over 90% must be immunized to stop its transmission (101). This is similar to the level of immunity required to interrupt the transmission of measles. In addition, eradication would require continuing immunity, which cannot be maintained with existing vaccine. Therefore eradication remains a distant goal.

Serotypes

The changes in the epidemiologic patterns in pertussis have occurred not only in relation to the host but also in relation to the parasite. Anderson demonstrated serological differences among *Bordetella* organisms, based on heat-labile agglutinogens, and Eldering and her colleagues extended these observations into an antigenic schema involving 14 agglutinogens (Table 3) (102,103). Agglutinogens unique to *B. pertussis* are factors 1 through 6. The serotype is usually identified by direct agglutination with monospecific antisera, but agglutinin production in animals may be a more accurate technique, particularly with the 1.2.3.4 serotype (104). Factor 1 is species-specific and the others may occur in various type-specific combinations. Serotypes appear to be stable if agglutinin production is used to determine the serotype, although serotype 1.2.3.4 may be a heterogenous group. When measured by direct agglutination, variation in serotype has been reported, even in the same patient (105).

Serotyping provides an epidemiologic marker which has been used to study changing patterns of *B. pertussis* over long periods. Serotypes in several western countries have changed from the 1.2.3 or 1.2 serotypes in the 1940s to the 1.3 serotype in the 1960s (Table 4) (106,107). The reason for the changes in serotypes is unknown but could be related to vaccine usage, if one postulates that vaccines select for the 1.3 serotype. Preston suggested

Table 4. Changing Patterns of *Bordetella pertussis* Serotypes

Country		Percent of Isolates by Decade		
	Serotype	1940s	1950s	1960s
United States [a]	1.3	38	85	95
	1.2.3	47	12	1
	1.2	5	3	2
United Kingdom [b]	1.3	4	8	73
	1.2.3	25	32	25
	1.2	67	60	1
Denmark [b]	1.3	—	32	48
	1.2.3	—	63	43
	1.2	—	5	3

[a] Eldering, G. et al.; reference 106
[b] Bronne-Shanbury, C. J. et al.; reference 107

that this shift occurred in the United Kingdom because the vaccine did not contain factor 3, and this was supported by the observation that the 1.3 serotype was isolated more frequently in vaccinated children and serotype 1.2.3 in unvaccinated children (83,108). An alternative explanation would be that the 1.2.3 serotype is not as effective an immunizing agent against the 1.3 serotype. Demina and colleagues have reported that vaccines containing the 1.2.3 serotype produce antibody against the 1.3 serotype in only 55% of vaccinees, but vaccine with 1.3 produces antibody to 1.2.3 in 80%. Also, the earlier introduction of effective vaccines in the United States, as compared to other countries, could explain the earlier shift from the 1.2.3 to the 1.3 serotype (Table 4). The best proof of the role of vaccine in altering the serotypes of *B. pertussis* would be the appearance of serotype 1.2 as the predominant serotype in countries using a vaccine lacking factor 2.

If vaccine has changed the prevalent serotype of *B. pertussis*, does this mean that the agglutinogens are protective antigens and are related to vaccine failures? Preston suggested that the absence of factor 3 in the United Kingdom vaccines contributed to the decreased vaccine efficacy in the 1960s (108). However, analysis of the agglutinogen content of American vaccines available in 1967 by Preston indicated that three of the four vaccines had no or very little factor 3 by slide agglutination, but there was no problem with vaccine efficacy (Table 5). The vaccines used in the United Kingdom during that period were less potent than the vaccines in the United States, and that, rather than the serotype content, probably accounted for the decreased efficacy. The effectiveness of vaccines in the United

Table 3. Bordetella Serotypes (Eldering)

Species	Characterizing Antigen or Factor		
	Genus	Species	Cultures Within Species
B. pertussis	7	1	2,3,4,5,6
B. parapertussis	7	14	8,9,10
B. bronchiseptica	7	12	8,9,10,11

Table 5. Agglutinogen Content of Pertussis Vaccines Available in the United States in 1967, as Determined by Slide Agglutination

Manufacturer	Agglutinogen Content		
	1	2	3
Parke-Davis	good	good	nil
Lederle	poor	good	poor
Lilly	poor	nil	good
Wyeth	nil	poor	nil

Figure 3. Serotypes of *B. pertussis* isolated from patients in New Orleans, Louisiana, 1967.

States suggests that the determination of serotype content of the vaccines was inaccurate or that serotype had no protective effect, or at least only a marginal one, which was compensated for by the total antigenic content of the vaccine. Animal studies using the passive protection procedure in mice have given conflicting results as to relationship between serotype and protection (109,110).

Serotyping has not been useful as an epidemiologic marker for studying individual epidemics, because of the limited number of serotypes. For instance, in a hospital outbreak in Cincinnati, Ohio, in 1974, all of the isolates from hospital-associated cases were serotypes 1.3.6, but so were the isolates from patients in the community who were not infected in the hospital (86). In contrast, in an epidemic in New Orleans in 1967, several serotypes were involved (Fig. 3) (48). There was no geographical clustering of serotypes, but there was an interesting temporal pattern. The predominant serotype was 1.3 throughout most of the epidemic period, but toward the end, the 1.2.3 serotype became more frequent. The reason for this apparent change in serotypes during an epidemic is not clear and has not been reported previously. In the British Public Health Laboratory Service studies, isolates obtained in the same household were serotyped by slide agglutination (83). In 21 of 198 households, isolates with and without factor 2 were identified in the same household, suggesting either that two strains of *B. pertussis* were involved or that the serotype had changed spontaneously. Bronne-Shanbury and Dolby have retested six of these pairs of isolates, using agglutinin production rather than direct agglutination (104). They were able to confirm only two pairs as showing different serotypes. The infrequency with which this occurs supports the concept of the stability of the serotype.

The clinical significance of the change in serotypes is also unknown. Demina and colleagues have
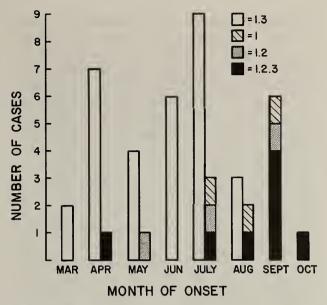
suggested that *B. pertussis* serotype 1.2.3 produces typical disease in 77% of infected patients and atypical disease in 23%, compared to serotype 1.3, which produces typical disease in 58%, and atypical disease in 42% (111). They define atypical disease as "difficult to make diagnosis without bacteriological confirmation," which presumably refers to disease without paroxysmal coughing. If this is true, then changing serotypes could have contributed to the changing severity of the disease. There are no other studies of the relationship of serotypes and clinical manifestations of disease to support this observation.

THE ROLE OF VIRUSES

An important question raised in recent years is whether or not other infectious agents, specifically viruses, are involved in the host-parasite interaction in pertussis. It took a long time for everyone to be convinced that pertussis was due to *B. pertussis* and not a virus. Experimental studies in animals and humans in the 1930s showed that *B. pertussis* alone was sufficient to cause pertussis and that a filtrate of nasopharyngeal washings or sputum would not (112,113). This did not exclude the possibilities that viruses may complicate infections by *B. pertussis* or may cause sporadic cases of a pertussislike syndrome.

Intranuclear inclusions were observed in the lungs of a patient with pertussis as early as 1927, and subsequently McCordock found similar inclusions in

the lungs of 12 of 35 cases of pertussis but in only 2 of 90 patients dying of other diseases (114). These and other reports described large cells with enlarged nuclei containing acidophilic intranuclear inclusions with a clear zone between the inclusion and the nuclear membrane. The chromatin was condensed along the nuclear membrane and, occasionally, cytoplasmic inclusions were seen. In 1950, McCordock's co-worker, Margaret G. Smith, reported that these inclusions represented what is now known as cytomegalovirus (CMV) and noted that the presence of CMV in one-third of cases of pertussis was unexplained (115). In view of current knowledge of CMV infections, it is possible that the virus is reactivated during pertussis. McCordock and Smith had noted inclusions in the salivary glands of 4 of 6 children with pertussis, in contrast to 6 of 60 children without pertussis (116). A recent study of nonfatal cases, using viral isolation to identify CMV infection, found CMV in 11% of children with the pertussis syndrome, but also in 18% of children with upper respiratory infections and 15% of well babies (117). However, 33% of children with pertussis who were less than 1 year of age were shedding CMV, compared to 15% of well babies. The pertussis cases in this study were not confirmed by culture, and the number of patients studied was too small to determine the significance of these observations, but this does suggest that pertussis may cause reactivation of latent CMV, or dissemination of primary CMV.

In 1932, Goodpasture reported finding inclusions similar to those described by McCordock, but he also described other inclusion bodies, which were probably due to adenovirus infection (118). Occasional cases of pertussis associated with adenovirus infection have been reported since then, but these received little attention until Olson and colleagues reported a family outbreak of pertussis associated with adenovirus type 12 infection (119). This was important because type 12 adenovirus is much rarer than the lower numbered types, which are commonly recovered from young children (120). Subsequently it was discovered that an error had been made in identifying the virus type, and the children had adenovirus type 2 rather than 12. Several studies of the relationship of adenoviruses and pertussis were reported in the 1970s (Table 6). These studies may be divided into three groups. The first group includes two studies which suggest that adenoviruses produce the pertussis syndrome (121, 122). The most disturbing study was that by Connor, reporting that 11 of 13 children with pertussis were infected with adenoviruses, including types 1, 2, 3, or 5. All the 13 children had negative cultures for *B. pertussis* and none of those tested had high levels of pertussis agglutinins. In evaluating this study and the one by Sturdy and colleagues it is important to note that the authors were unable to diagnose *B. pertussis* in any patients. In the second group of studies, involving over 800 children, the investigators were able to recover *B. pertussis* from 21–49% of the patients, and adenoviruses from 8–10% (123–126). Although adequate controls were not included, all of these studies showed that the recovery of adenoviruses was the same in patients with positive cultures for *B. pertussis* and those with negative cultures. If adenoviruses were causing pertussis in a significant number of children, the recovery rate might be expected to be greater in culture-negative cases. The third group of studies includes a report by Klenk and colleagues (127) which contrasts with those by Connor and Sturdy et al. in that *B. pertussis* was isolated from the majority of patients. They also recovered adenoviruses from one-third of the pa-

Table 6. The Relationship of Adenoviruses and the Pertussis Syndrome

| Study | No. with Pertussis Syndrome | Type of Infection | | | Adenovirus Types |
		Adeno-virus	*B. pertussis*	Both	
I Connor 1970	13	11(85%)	0	0	1,2,3,5
Sturdy et al. 1971	34	5(15%)	0	0	1,2,5,7
II Calder et al. 1970	210	16(8%)	102(49%)	9(4%)	Not given
Pereira & Candeias 1971	136	11(8%)	29(21%)	3(2%)	1,2,3,5
Lewis et al. 1973	483	49(10%)	175(36%)	23(5%)	1,2,5,6,7,9
III Klenk et al. 1972	19	7(37%)	14(74%)[a]	7(37%)	1,5
Nelson et al. 1975	134	30(22%)	46(34%)	18(13%)	1,2,12

[a] Includes one *B. parapertussis* infection

tients. Virus was recovered from 7 of 14 patients with culture-proven *B. pertussis* infections but from none of 5 with culture-negative pertussis. The other study, reported by Nelson and colleagues, included a control population (128). They reported that adenoviruses were recovered significantly more often in patients with culture-proven *B. pertussis* infections than in either patients with culture-negative pertussis or normal controls. The authors did not observe a difference in severity in those with or without positive bacterial or viral cultures. These studies suggest that *B. pertussis* infection may cause reactivation of adenovirus infection.

The question still remains as to whether or not adenoviruses cause sporadic cases of pertussislike illness. Part of the answer depends on the definitions used. Adenoviruses are known to cause paroxysmal coughing, but the pertussis syndrome is usually defined as paroxysmal coughing with an inspiratory whoop and/or lymphocytosis. Chany and colleagues described a series of 23 children with adenovirus infection and paroxysmal coughing, but only one of them had lymphocytosis (129). This child had a clinical diagnosis of whooping cough with pneumonia, and his was the only case which was not confirmed as an adenovirus infection by either culture or antibody rise. Large epidemiological studies of adenoviruses and clinical illness have failed to identify pertussis as part of the clinical spectrum. In a study involving 18,096 children, adenoviruses were recovered from 1,796 (10%) (120). Types 1, 2, 3, and 5 accounted for most of the isolates. This is similar to the experience in children with pertussis (Table 6). Therefore, although adenoviruses may frequently cause paroxysmal coughing, when an inspiratory whoop and lymphocytosis are present in a patient with an adenovirus infection the possibility of a dual infection with *B. pertussis* should be considered. In many medical centers, it is easier to culture adenoviruses than *B. pertussis*.

CONCLUSION

The basic aspects of the host-parasite interaction in pertussis, both in the individual and the community, have been reviewed with particular emphasis on the effects of immunization. The latter include changes in the severity of disease, the age distribution of cases, the prevalent serotypes of *B. pertussis*, and, at least in part, the decrease in incidence and mortality. This review has emphasized

the questions which need further clarification, including the following:

1. Which of the biologically active components or properties of *B. pertussis* have a role in the pathogenesis of disease in man?
2. What is the mechanism of cell injury after attachment of *B. pertussis* to ciliated epithelial cells?
3. How does *B. pertussis* produce the prolonged paroxysmal cough characteristic of pertussis?
4. What is the role of the local immune response in the respiratory tract in recovery from and resistance to pertussis?
5. How frequently does clinically unrecognized infection occur in vaccinated populations?
6. How do existing vaccines protect, and what is the continuing role of vaccine in the changing host-parasite interactions in pertussis?

An understanding of the pathogenesis and epidemiology of a disease is not absolutely necessary for control of the disease. However, such an understanding does facilitate progress toward that goal and may be particularly important in a disease such as pertussis. Pertussis appears to be subdued by immunization but still persists in the community. If routine immunization were discontinued the disease might reemerge as a major public health problem.

REFERENCES

1. Sydenham, T. Opera omnia. Greenhill, W. A., ed. London: Sydenham Society; 1846: 187.
2. Baillou, G. Constitutio aestiva, Anni Domini 1578. Major, R.H., ed. Classic descriptions of disease, 3rd edition. Springfield, Illinois: Charles C Thomas; 1945: 210–212.
3. Olson, L. C. Pertussis. Medicine 54:427–469; 1975.
4. von Rosenstein, N. R. On hooping cough. Clendening, L. ed. Source book of medical history. New York: Dover Publications; 1960: 266–268.
5. Creighton, C. A history of epidemics in Britain. Cambridge: University Press; 1894: 666–677.
6. Sticker, G. Whooping cough. Moore, J. W., ed. Variola, vaccination, varicella, cholera, erysipelas, whooping cough, hay fever. Philadelphia and London: W. B. Saunders; 1902: 539–618.
7. Radbill, S. X. Whooping cough in fact and fancy. Bull. Hist. Med. 13:33–53; 1943.
8. Bordet, J.; Gengou, O. Le microbe de la coqueluche. Ann. Inst. Pasteur 20:731–741; 1906.
9. Bradford, W. L.; Day, E.; Berry, G. P. Improvement of the nasopharyngeal swab method of diagnosis in pertussis by the use of penicillin. Am. J. Public Health 36:468–470; 1946.
10. Linnemann, C. C.; Partin, J. C.; Perlstein, P. H.; Englender, G. S. Pertussis: persistent problems. J. Pediatr. 85:589–591; 1974.

11. Pittman, M. Genus *Bordetella*. Buchanan, R. E.; Gibbons, N. E., eds. Bergey's manual of determinative bacteriology, 8th edition. Baltimore: Williams & Wilkins Co.; 1974: 282–283.

12. Kumazawa, N. H.; Yoshikawa, M. Conversion of *Bordetella pertussis* to *Bordetella parapertussis* J. Hyg. (Camb.) 81:15–23; 1978.

13. Morse, J. H.; Morse, S. I. Studies on the ultrastructure of *Bordetella pertussis*. I. Morphology, origin, and biological activity of structures present in the extracellular fluid of liquid cultures of *Bordetella pertussis*. J. Exp. Med. 131:1342–1357; 1970.

14. Morse, S. I. Biologically active components and properties of *Bordetella pertussis*. Adv. Appl. Microbiol. 20: 9–26; 1976.

15. Munoz, J. J.; Bergman, R. K. *Bordetella pertussis*. Immunological and other biological activities. New York and Basel: Marcel Dekker, Inc.; 1977.

16. Morse, S. I.; Riester, S. K. Studies on the leukocytosis and lymphocytosis induced by *Bordetella pertussis*. I. Radioautographic analysis of the circulating cells in mice undergoing pertussis-induced hyperleukocytosis. J. Exp. Med. 125:401–408; 1967.

17. Morse, S. I.; Riester, S. K. Studies on the leukocytosis and lymphocytosis induced by *Bordetella pertussis*. II. The effect of pertussis vaccine on the thoracic duct lymph and lymphocytes of mice. J. Exp. Med. 125:619–628; 1967.

18. Morse, S. I.; Baron, B. A. Studies on the leukocytosis and lymphocytosis induced by *Bordetella pertussis*. III. The distribution of transfused lymphocytes in pertussis-treated and normal mice. J. Exp. Med. 132:663–672; 1970.

19. Morse, S. I. Lymphocytosis-promoting factor of *Bordetella pertussis*: isolation, characterization and biological activity. J. Infect. Dis. 136:S234–S238; 1977.

20. Hewlett, E. L.; Urban, M. A.; Manclark, C. R.; Wolff, J. Extracytoplasmic adenylate cyclase of *Bordetella pertussis*. Proc. Natl. Acad. Sci. U.S.A. 73:1926–1930; 1976.

21. Mallory, F. B.; Hornor, A. A. Pertussis: the histological lesion in the respiratory tract. J. Med. Res. 27:115–123; 1912.

22. Mallory, F. B.; Hornor, A. A.; Henderson, F. F. The relation of the Bordet-Gengou bacillus to the lesion of pertussis. J. Med. Res. 27:391–397; 1913.

23. Mallory, F. B. The pathological lesion of whooping cough. Boston Med. Surg. J. 169:378–380; 1913.

24. Collier, A. M.; Peterson, L. P.; Baseman, J. B. Pathogenesis of infection with *Bordetella pertussis* in hamster tracheal organ culture. J. Infect. Dis. 136:S196–S203; 1977.

25. Muse, K. E.; Collier, A. M.; Baseman, J. B. Scanning electron microscopic study of hamster tracheal organ cultures infected with *Bordetella pertussis*. J. Infect. Dis. 136:768–777; 1977.

26. Matsuyama, T. Resistance of *Bordetella pertussis* phase 1 to mucociliary clearance by rabbit tracheal mucous membrane. J. Infect. Dis. 136:609–616; 1977.

27. Bemis, D. A.; Greisen, H. A.; Appel, M. J. G. Pathogenesis of canine bordetellosis. J. Infect. Dis. 135:753–762; 1977.

28. Holt, L. B. The pathology and immunology of *Bordetella pertussis* infection. J. Med. Microbiol. 5:407–424; 1972.

29. Smith, L. W. The pathologic anatomy of pertussis with especial reference to pneumonia caused by the pertussis bacillus. Arch. Pathol. 4:732–742; 1927.

30. Lapin, J. H. Whooping cough. Springfield, Ill.: Charles C Thomas; 1943.

31. Dolgopol, V. B. Changes in the brain in pertussis with convulsions. Arch. Neurol. Psychiatr. 46:477–503; 1941.

32. Court, D.; Jackson, H.; Knox, G. The recognition of whooping-cough. Lancet 2:1057–1060; 1953.

33. Thelander, H. E.; Henderson, H. G.; Kilgariff, K. The blood picture in pertussis, a graphic study. J. Pediatr. 2:288–298; 1933.

34. Lagergren, J. The white blood cell count and the erythrocyte sedimentation rate in pertussis. Acta Paediatr. 52:405–409; 1963.

35. Regan, J. C.; Tolstoouhov, A. Relations of acid base equilibrium to the pathogenesis and treatment of whooping cough. N.Y. State J. Med. 36:1075–1087; 1936.

36. Collins, S. D. Age incidence of the common communicable diseases of children. Public Health Rep. 44:763–826; 1929.

37. Vysoka, B. The epidemiology of pertussis and parapertussis. J. Hyg. (Camb.) Epidemiol. Microbiol. Immunol. II:196–204; 1958.

38. Madsen, T. Lectures on the epidemiology and control of syphilis, tuberculosis and whooping cough, and other aspects of infectious disease. Baltimore: Williams & Wilkins Co.; 1937.

39. Bassili, W. R.; Stewart, G. T. Epidemiological evaluation of immunisation and other factors in the control of whooping cough. Lancet 1:471–473; 1976.

40. Kendrick, P.; Eldering, G. A study in active immunization against pertussis. Am. J. Hyg. (Sect B) 29:133–153; 1939.

41. Miller, C. L.; Fletcher, W. B. Severity of notified whooping cough. Br. Med. J. 1:117–119; 1976

42. Congeni, B. L.; Orenstein, D. M.; Nankervis, G. A. Three infants with neonatal pertussis. Clin. Pediatr. 17:113–118; 1978.

43. Gordon, J. E.; Hood, R. I. Whooping cough and its epidemiological anomalies. Am. J. Med. Sci 222:333–361; 1951

44. Linnemann, C. C.; Nasenbeny, J. Pertussis in the adult. Ann. Rev. Med. 28:179–185; 1977.

45. Morley, D.; Woodland, M.; Martin, W. J. Whooping cough in Nigerian children. Trop. Geogr. Med. 18:169–182; 1966.

46. Brooks, A. M.; Bradford, W. L.; Berry, G. P. The method of nasopharyngeal culture in the diagnosis of whooping cough. J.A.M.A. 120:883–885; 1942.

47. Miller, J. J.; Leach, C. W.; Saito, T. M.; Humber, J. B. Comparison of the nasopharyngeal swab and the cough plate in the diagnosis of whooping cough and *Hemophilus pertussis* carriers. Am. J. Public Health 33:839–843; 1943.

48. Bass, J. W.; Klenk, E. L.; Kotheimer, J. B.; Linnemann, C. C.; Smith, M. H. D. Antimicrobial treatment of pertussis. J. Pediatr. 75:768–781; 1969.

49. Wanner, A. Clinical aspects of mucociliary transport. Am. Rev. Resp. Dis. 116:73–125; 1977.

50. Pittman, M. *Bordetella pertussis*—bacterial and host factors in the pathogenesis and prevention of whooping cough. Mudd, S., ed. Infectious agents and host reactions. Philadelphia: W. B. Saunders Co.; 1970: 239–270.

51. Pavesio, D.; Ponzone, A. Salbutamol and pertussis (letter). Lancet 1:150–151; 1977.

52. McKendrick, G. D. W.; Raychoudhury, S. C. Management of whooping cough (letter). Lancet 2:1083; 1977.

53. Zoumboulakis, D.; Anagnostakis, D.; Albanis, V.; Matsaniotis, N. Steroids in treatment of pertussis. A controlled clinical trial. Arch. Dis. Child. 48:51–54; 1973.

54. Kaufman, S.; Bruyn, H. B. Pertussis, a clinical study. Am. J. Dis. Child. 99:417-422; 1960

55. Brooksaler, F.; Nelson, J. D. Pertussis. A reappraisal and report of 190 confirmed cases. Am. J. Dis. Child. 114: 389–396; 1967.
56. White, R.; Finberg, L.; Tramer, A. The modern morbidity of pertussis in infants. Pediatrics 33:705–710; 1964.
57. Jernelius, H. Pertussis with pulmonary complications: a follow-up study. Acta Paediatr. Scand. 53:247–254; 1964.
58. Celermajer, J. M.; Brown, J. The neurological complications of pertussis. Med. J. Aust. 1:1066–1069; 1966.
59. Harper, J. R.; Maguire, M. J. Hyponatremia and convulsions in whooping cough (letter). Lancet 1:1147; 1975.
60. Laing J. S.; Hay, M. Whooping-cough: Its prevalence and mortality in Aberdeen. Public Health 14:584–599; 1902.
61. Horner, F. A. Second attack of pertussis (letter). N. Engl. J. Med. 266: 470–471; 1962.
62. Huang, C. C.; Chen, P. M.; Kuo, J. K.; Chiu, W. H.; Lin, S. T.; Lin, H. S.; Lin, Y. C. Experimental whooping cough. N. Engl. J. Med. 266:105–111; 1962.
63. Gray, D. F.; Cheers, C. The steady state in cellular immunity. II. Immunological complaisance in murine pertussis. Aust. J. Exp. Biol. Med. Sci. 45:417–426; 1967.
64. Cheers, C.; Gray, D. F. Macrophage behaviour during the complaisant phase of murine pertussis. Immunology 17:875–887; 1969.
65. Gray, D. F.; Cheers, C. The sequence of enhanced cellular activity and protective humoral factors in murine pertussis immunity. Immunology 17: 889–896; 1969.
66. Donald, A. B. The diagnosis of whooping cough. Br. Med. J. 3:613–618; 1938.
67. Miller, J. J.; Silverberg, R. J. The agglutinative reaction in relation to pertussis and to prophylactic vaccination against pertussis with description of a new technique. J. Immunol. 37:207–219; 1939.
68. Aftandelians, R.; Connor, J. D. Bactericidal antibody in serum during infection with Bordetella pertussis. J. Infect. Dis. 128:555–558; 1973.
69. Neel, J. V.; Andrade, A. H. P.; Brown, G. E.; Eveland, W. E.; Goobar, J.; Soderman, W. A.; Stollerman, G. H.; Weinstein, E. D.; Wheeler, A. H. Further studies of the Xavante Indians. IX. Immunological status with respect to various diseases and organisms. Am. J. Trop. Med. Hyg. 17:486–498; 1968.
70. Aftandelians, R.; Connor, J. D. Immunologic studies of pertussis. Development of precipitins. J. Pediatr. 83:206–214; 1973.
71. Thomas, G. Respiratory and humoral immune response to aerosol and intramuscular pertussis vaccine. J. Hyg. (Camb.) 74:233–237; 1975.
72. Combined Scottish Study. Diagnosis of whooping cough: comparison of serological tests with isolation of Bordetella pertussis. Br. Med. J. 4:637–639; 1970.
73. Morris, D.; McDonald, J. C. Failure of hyperimmune gamma globulin to prevent whooping cough. Arch. Dis. Child. 32:236–239; 1957.
74. Balagtas, R. C.; Nelson, K. E.; Levin, S.; Gotoff, S. P. Treatment of pertussis with pertussis immune globulin. J. Pediatr. 79:203–208; 1971.
75. Miller, J. J.; Silverberg, R. J.; Saito, T. M.; Humber, J. B. An agglutinative reaction for Hemophilus pertussis. II. Its relation to clinical immunity. J. Pediatr. 22:644–651; 1943.
76. Sako, W.: Studies on pertussis immunization. J. Pediatr. 30:29–40; 1947.
77. Geller, B. D.; Pittman, M. Immunoglobulin and histamine-sensitivity response of mice to live Bordetella pertussis. Infect. Immun. 8:83–90; 1973.
78. Bernales, R.; Eastman, J.; Kaplan, J. Quantitation of circulating T and B lymphocytes in children with whooping cough. Pediatr. Res. 10:965–967; 1976.
79. Pieroni, R. E.; Stevens, D. L.; Stojanovic, A.; Levine, L. Investigation of responsiveness of BCG-vaccinated children with whooping-cough to tuberculin. Int. Arch. Allergy Appl. Immunol. 42:583–589; 1972.
80. Cheers, C. Delayed hypersensitivity in murine pulmonary pertussis. Aust. J. Exp. Biol. Med. Sci. 47:513–520; 1969.
81. Linnemann, C. C.; Bass, J. W.; Smith, M. H. D. The carrier state of pertussis. Am J. Epidemiol. 88:422–427; 1968.
82. Borska, K.; Simkovicova, M. Studies on the circulation of Bordetella pertussis and Bordetella parapertussis in populations of children. J. Hyg. Epidemiol. Microbiol. Immunol. 16:159–172; 1972.
83. Public Health Laboratory Service. Efficacy of whooping-cough vaccines used in the United Kingdom before 1968. Br. Med. J. 1:259–262; 1973.
84. Luttinger, P. The epidemiology of pertussis. Am. J. Dis. Child. 12:290–315; 1916.
85. Nelson, J. D. The changing epidemiology of pertussis in young infants. The role of adults as reservoirs of infection. Am. J. Dis. Child. 132:371–373; 1978.
86. Linnemann, C. C.; Ramundo, N; Perlstein, P. H.; Minton, S. D.; Englender, G. S. Use of pertussis vaccine in an epidemic involving hospital staff. Lancet 2:540–543; 1975.
87. Kurt, T. L.; Yeager, A. S.; Guenette, S.; Dunlop, S. Spread of pertussis by hospital staff. J.A.M.A. 221:264–267; 1972.
88. Culotta, C. E.; Dominick, D.; Harrison, E. R. Epidemiologic studies in whooping cough. Yale J. Biol. Med. 10:473–483; 1938.
89. Rodman, A. C.; Bradford, W. L.; Berry, G. P. An epidemiological study of an outbreak of pertussis in a public school. Am. J. Public Health 36:1156–1162; 1946.
90. Stocks, P. Some epidemiological features of whooping-cough. A statistical investigation. Lancet 1:265–269; 1933.
91. Macdonald, H.: Macdonald, E. J. Experimental pertussis. J. Infect. Dis. 53:328–330; 1933.
92. Bell, J. A. Whooping cough. Sartwell, P. E., ed. Preventive medicine and public health, 9th edition. New York: Appleton-Century Crofts; 1965: 189–198.
93. Ipsen, J.; Bowen, H. E. Effects of routine immunization of children with triple vaccine (diphtheria-tetanus-pertussis). Am. J. Public Health 45:312–318; 1955.
94. Vysoka-Burianova, B. Contemporary problems in the epidemiology of whooping cough. J. Hyg. Epidemiol. Microbiol. Immunol. 7:472–480; 1963.
95. Morse, S. I. Pertussis in adults (editorial). Ann. Intern. Med. 68:953–954; 1968.
96. Lambert, H. J. Epidemiology of a small pertussis outbreak in Kent County, Michigan. Public Health Rep. 80:365–369; 1965.
97. Miller, C. L.; Pollock, T. M.; Clewer, A. D. E. Whooping-cough vaccination. An assessment. Lancet 2:510–513; 1974.
98. Stewart, G. T. Vaccination against whooping-cough. Efficacy versus risks. Lancet 1:234–237; 1977.
99. Jenkinson, D. Outbreak of whooping cough in general practice. Br. Med. J. 3:577–578; 1978.
100. Kendrick, P. L. Can whooping cough be eradicated? J. Infect. Dis. 132:707–712; 1975.
101. Zakharova, M. S.; Novikova, O. M.; Yermakov, S. P.; Sukhov, Y. V. An attempt to correlate whooping cough frequency rates with proportion of vaccinated in

a child population J. Hyg. Epidemiol. Microbiol. Immunol. 21:128–135; 1977.

102. Andersen, E. K. Serological studies on *H. pertussis, H. parapertussis* and *H. bronchisepticus.* Acta Pathol. Microbiol. Scand. 33:202–224; 1953.

103. Eldering, G.; Hornbeck, C.; Baker, J. Serological study of *Bordetella pertussis* and related species. J. Bacteriol. 74:133–136; 1957.

104. Bronne-Shanbury, C. J.; Dolby, J. M. The stability of the serotypes of *Bordetella pertussis* with particular reference to serotype 1,2,3,4. J. Hyg. (Camb.) 76:277–286; 1976.

105. Zakharova, M. S.; Gorbunova, Z. V.; Kuznetsov, E. A.; Denisova, T. P. The epidemiological situation in various countries. Open discussion. van Hemert, P. A.; van Ramshorst, J. D.; Regamey, R. H., eds. Symposia series in immunobiological standardisation no. 13. International symposium on pertussis, Basel, Munich, New York: Karger; 1970: 34–35.

106. Eldering, G.; Holwerda, J.; Davis, A.; Baker, J. *Bordetella pertussis* serotypes in the United States. Appl. Microbiol. 18:618–621; 1969.

107. Bronne-Shanbury, C. J.; Miller, D.; Standfast, A. F. B. The serotypes of *Bordetella pertussis* isolated in Great Britain between 1941 and 1968 and a comparison with the serotypes observed in other countries over this period. J. Hyg. (Camb.) 76:265–275; 1976.

108. Preston, N. W. Type-specific immunity against whooping cough. Br. Med. J. 3:724–726; 1963.

109. Preston, N. W. Potency tests for pertussis vaccines: doubtful value of intracerebral challenge test in mice. J. Pathol. Bacteriol. 91:173–179; 1966.

110. Eldering, G.; Holwerda, J.; Baker, J. Mouse-protective properties of *Bordetella pertussis* serotypes in passive tests. J. Bacteriol. 93:1758–1761; 1967.

111. Demina, A. A.; Devyatkina, N. P.; Voloshina, L. Z.; Larina, L. I.; Semina, N. H.; Venglinskaya, E. A.; Kostyukova, K. P. Serological types of whooping cough bacteria and their connection with immunity in vaccinated children. J. Hyg. Epidemiol. Microbiol. Immunol. 17:304–315; 1973.

112. Rich, A. R.; Long, P. H.; Brown, J. H.; Bliss, E. A.; Holt, L. E. Experiments upon the cause of whooping cough. Science 76:330–331; 1932.

113. Frawley, J. M. A study of the virus factor in whooping cough. J. Pediatr. 16:18–20; 1940.

114. McCordock, H. A. Intranuclear inclusions in pertussis. Proc. Soc. Exp. Biol. Med. 29:1288–1291; 1932.

115. Smith, M. G.; Vellios, F. Inclusion disease or generalized salivary gland virus infection. Arch. Pathol. 50:862–884; 1950.

116. McCordock, H. A.; Smith, M. G. Intranuclear inclusions. Incidence and possible significance in whooping cough, and in a variety of other conditions. Am. J. Dis. Child. 47:771–779; 1934.

117. Olson, L. C.; Miller, G.; Hanshaw, J. B. Acute infectious lymphocytosis presenting as a pertussis-like illness: Its association with adenovirus type 12. Lancet I:200–201; 1964.

118. Goodpasture, E. W.; Auerbach, S. H.; Swanson, H. S.; Cotter, E. F. Virus pneumonia of infants secondary to epidemic infections Am. J. Dis. Child. 57:997–1011; 1939.

119. Olson, L. C.; Ketusinha, R.; Mansuwan, P.; Snitbham, R. Respiratory tract excretion of cytomegalovirus in Thai children. J. Pediatr. 77:499–504; 1970.

120. Brandt, C. D.; Kim, H. W.; Vargosko, A. J.; Jeffries, B. C.; Arrobio, J. O.; Rindge, B.; Parrott, R. H.; Chanock, R. M. Infections in 18,000 infants and children in a controlled study of respiratory tract disease. I. Adenovirus pathogenicity in relation to serologic type and illness syndrome. Am. J. Epidemiol. 90:484–500; 1969.

121. Connor, J. D. Evidence for an etiologic role of adenoviral infection in pertussis syndrome. N. Engl. J. Med. 283:390–394; 1970.

122. Sturdy, P. M.; Court, S. D. M.; Gardner, P. S. Viruses and whooping-cough (letter). Lancet 2:978–979; 1971.

123. Calder, M. A.; Duguid, J. P.; Elias-Jones, T. F.; Fallon, R. J.; Gillies, R. R.; Green, D. M.; Grist, N. R.; Lawson, J. H.; Love, W. C.; McKenzie, P.; Pinkerton, I. W. R.; Constance, A. C.; Sangster, G.; Wilson, A. M. M. What causes whooping-cough? (letter). Lancet 2:1079; 1970.

124. Pereira, M. S.; Candeias, J. A. N. The association of viruses with clinical pertussis. J. Hyg. (Camb.) 69:399–403; 1971.

125. Bennett, N. McK. Whooping cough in Melbourne. Med. J. Aust. 2:481–487; 1973.

126. Lewis, F. A.; Gust, I. D.; Bennett, A. McK. On the aetiology of whooping cough. J. Hyg (Camb.) 71:139–144; 1973.

127. Klenk, E. L.; Gaultney, J. V.; Bass, J. W. Bacteriologically proven pertussis and adenovirus infection. Possible association. Am. J. Dis. Child. 124:203–207; 1972.

128. Nelson, K. E.; Gavitt, F.; Batt, M. D.; Kallick, C. A.; Reddi, K. T.; Levin, S. The role of adenoviruses in the pertussis syndrome. J. Pediatr. 86:335–341; 1975.

129. Chany, C.; Lepine, P.; Lelong, M.; Le-tan-Vinh; Satge, P.; Virat, J. Severe and fatal pneumonia in infants and young children associated with adenovirus infections. Am. J. Hyg. 67:367–378; 1958.

Pertussis—Diagnostic Methods and Surveillance

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ABSTRACT

Simultaneous use of direct immunofluorescence and cultures directly plated from nasopharyngeal swabs obtained during an outbreak of pertussis in Atlanta 1977 demonstrated that culture, in addition to being specific, was more sensitive than direct immunofluorescence for diagnosis. Thirty of 36 (83%) cases were identified by culture as compared with only 22 (61%) by direct immunofluorescence when the two techniques were used in parallel. In the same trial, methicillin was found to be more satisfactory than penicillin as a selective agent in Bordet-Gengou medium. During this outbreak we found four asymptomatic culture-positive individuals who remained asymptomatic during 4 weeks of followup. Analysis of observer variation in an investigation of pertussis in Oregon showed that direct immunofluorescence testing had poor specificity under certain conditions.

A national surveillance program for pertussis, which will begin in 1978, is designed to provide information on methods of diagnosis, morbidity, mortality, vaccine efficacy, and antibiotic prophylaxis.

INTRODUCTION

Formulation of sound pertussis control policy requires accurate information about the occurrence, spread, and severity of the disease and the impact of control measures. Accurate diagnosis is an essential starting point. However, the majority of cases called "pertussis" are based on clinical diagnosis alone, despite clear evidence that *B. parapertussis* and adenoviruses can cause whooping cough and that many infections with *B. pertussis* may be atypical. Recent outbreaks of pertussis in Atlanta, Georgia, and Portland, Oregon, gave us the opportunity to reevaluate diagnostic methods by comparing the sensitivity and specificity of optimal culture techniques and direct immunofluorescence. Working with the Conference of State and Territorial Epidemiologists, we have initiated national surveillance of pertussis to gather additional data needed for policy decisions and to analyze the data on the basis of method of diagnosis.

METHODS

Comparison of Culture and Direct Immunofluorescence

Patient Selection. Nasopharyngeal (NP) swabs were obtained from patients with clinically suspect pertussis and contacts of such cases in the patient population of a large metropolitan hospital in Atlanta between April 1 and September 20, 1977.

Cases were identified predominantly through the outpatient pediatric emergency clinic.

Culture. In the initial phase of the outbreak (April 1 to August 17, 1977), calcium alginate nasopharyngeal swabs from patients were placed in a modified Stuart's transport medium (Trans-cul, Wampole Laboratories) and sent to the hospital's central bacteriology laboratory. After a period that varied from 15 minutes to several hours, the swabs were used to inoculate two plates of Bordet-Gengou (BG) medium freshly prepared at the hospital from BG agar base (Nolan Biologicals), supplemented with 15% fresh defibrinated sheep's blood; agar in one plate was supplemented with 0.5 unit of penicillin per milliliter, and one plate was without penicillin. Microscope slides for testing by direct immunofluorescence were also prepared at this time. The plates were incubated at the hospital for up to 24 hours at 35° C until the plates and slides were sent to the Center for Disease Control (CDC) for interpretation.

During the second phase (August 18 to September 20, 1977), BG medium was freshly prepared by the CDC laboratory from a BG agar base, (Difco Laboratories), to which was added 1% glycerol and 15% fresh defibrinated sheep's blood. Half the plates were made with media containing 0.5 unit of penicillin per ml and half contained 2.5 μg methicillin per ml. House officers were instructed to prepare slides for direct immunofluorescence

testing and to inoculate the plates directly from the calcium alginate NP swabs without using transport media. For each child, one penicillin plate and one methicillin plate were inoculated. Inoculated plates were streaked in the hospital bacteriology laboratory, incubated at 35° C in a moist atmosphere and sent within 24 hours to the CDC, where incubation resumed. They were examined daily under a dissecting microscope for colonies with typical appearance and surrounding hemolysis. Suspicious colonies were verified as *B. pertussis* by direct immunofluorescence.

Direct Immunofluorescence. Antisera against *B. pertussis* and *B. parapertussis* were prepared and conjugated according to the procedures of Hebert et al. (1). All NP smears were stained for 30 minutes with the conjugate against *B. pertussis* and the conjugate against *B. parapertussis.* Slides were examined on a Leitz Dialux microscope, using a 50 W halogen lamp, a monocular head, a Ploem incident illuminator equipped for primary excitation with 2 KP 490 interference filters and a TK 510 dichroic beam splitting mirror coupled with a K 515 suppression filter. An ocular suppression filter was not needed.

Comparative Reading of Direct Immunofluorescence Slides

Patient Selection. Twenty consecutive specimens submitted to the Oregon State Health Department Laboratory for direct immunofluorescence testing for pertussis were selected. Of the ten in which a clinical history was available, seven were taken from patients with clinically suspect pertussis and three were from contacts of such cases.

Direct Immunofluorescence. The conjugate used was prepared at the CDC as described above. Slides were stained at the Oregon Laboratory, using a 1:8 dilution of the conjugate, and were read that day by the two usual readers, neither of whom was aware of the other's interpretation. Slides were examined on a Leitz Wetzlar microscope, using an HBO 200 mercury lamp with a monocular head. The exciter filter used was BG12, and the barrier filter was OG1.

Slides were then sent to the CDC, where they were examined according to the method previously described by a reader who was unaware of the results of the other readings.

Surveillance Data

Surveillance data were based on notifications by state health departments to CDC of all pertussis cases reported to them. Ages of patients were reported for the first time in 1977, if that information was available to the state health department.

RESULTS

Comparison of culture and direct immunofluorescence for identification of *B. pertussis* was possible in 91 individuals who had the two tests performed in parallel. The results of this comparative testing can be seen in Table 1. During the earlier phase, which differs from the later phase primarily in the use of Stuart's transport medium, culture was significantly less sensitive than direct immunofluorescence (36% versus 100%, p <0.0001, McNemar test). However, when swabs were directly inoculated on BG media, culture was at least as sensitive as direct immunofluorescence (83% versus 61%, 0.05< p <0.10, McNemar test).

Thirty cultures were plated on both the penicillin- and methicillin-containing BG media; 24 pairs were positive on both plates, 2 pairs were positive only on the penicillin plate, and 4 pairs were positive only on the methicillin plate. Cultures on the methicillin plate were subjectively easier to interpret because of better suppression of the growth of other pharyngeal flora.

Four of the positive cultures were taken from asymptomatic individuals. Three of them were children between the ages of 2 and 4 years, and one was a 26-year-old. Two of the children had had four DTP immunizations and one had had three. One of the children had been exposed to a case of culture-positive pertussis, beginning 1 month before her positive culture was obtained. All asymptomatic individuals were treated with erythromycin within 4 days of the date the culture was taken; none developed symptoms suggestive of pertussis in the 4 weeks following the positive culture.

Table 2 shows the results of the comparison of immunofluorescence readings of different observers. All three readers agreed on only 10 of the 25 slides (2 positives and 8 negatives). Readers A to B and

Table 1. Comparison of Culture and Direct Immunofluorescence for Identification of *B. pertussis*, Atlanta, 1977

Culture Method[a]	Culture +, FA +	Culture +, FA −	Culture −, FA +	Total
Phase I	20	0	35	55
Phase II	16	14	6	36
Total	36	14	41	91

[a] See text for details.

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Table 2. Comparison of Results of Direct Immuno-
fluorescence for *B. pertussis* on NP Smears Among
Three Readers

	Reader		
Smear Number	A	B	C
1	+	−	+
2	+	−	−
3	+	+	−
4	−	−	−
5	−	−	−
6	−	+	+
7	+	−	+
8	−	−	+
9	−	−	+
10	−	−	−
11	−	−	−
12	−	−	−
13	+	+	+
14	−	+	+
15	−	+	+
16	−	−	−
17	−	−	+
18	+	+	+
19	−	−	−
20	−	−	−

Table 3. Age Distribution of Reported Pertussis Cases,
United States, 1977

Age (years)	No. of Cases	%
<1	680	28.0
1–4	545	25.0
5–9	214	9.8
10–14	144	6.7
15–19	58	2.7
20–24	22	1.0
25–29	27	1.2
30–39	40	1.8
40–49	9	0.4
50–59	4	0.2
60+	4	0.2
Unknown	500	23.0
Total	2177	100

readers B to C showed agreement on 14 slides;
readers A and C agreed on 12 slides.

Table 3 shows the age distribution of reported
cases of pertussis throughout the United States in
1977; 28% of cases occurred in children less than
1 year of age.

DISCUSSION

Attempts to confirm the diagnosis of pertussis by
laboratory methods have focused upon either cul-
ture or direct immunofluorescence. Previous studies
of the comparative sensitivity of the two methods
have been done by Brooksaler and Chalvardjian,
both of whom found increased sensitivity with di-
rect immunofluorescence; Holwerda, who showed
no difference between the two techniques; and
Field, whose data suggested a slightly increased
sensitivity with culture (2–5). Our data confirm the
finding that culture is at least as sensitive as direct
immunofluorescence when plates are directly in-
oculated from the NP swab. The finding that use
of Stuart's transport medium appeared to decrease
the sensitivity of culture is consistent with findings
of Jones (6).

Use of methicillin as a selective agent in the BG
media also appears to improve the sensitivity of
culture. Chalvardjian, Baraff, and Field have also
commented on an improved ability to isolate *B.
pertussis* with the use of methicillin as a selective
agent (3,5,7).

The sensitivity of culture for identification of
B. pertussis is affected by the amount of time be-
tween disease onset and obtaining the culture, and
the prior use of antibiotics known to eradicate *B.
pertussis* from the nasopharynx (7,9). Other factors
which may result in variation in culture results
include the age and immunization status of the
patient (7).

The evidence of equal or improved sensitivity of
culture as compared to direct immunofluorescence
is in addition to the benefit of 100% specificity.
This is in contrast to direct immunofluorescence,
for which estimates of false positive results ranged
from 6.7% to 40% (4,12). The potential problems
with direct immunofluorescence are underlined by
the variability between readers under some circum-
stances (Table 2). We have not identified the exact
source of the variability. The delay involved in
shipping specimens from Oregon to Atlanta may
have resulted in some loss of fluorescence, but this
could only explain negative results in Atlanta
(Reader A) when the Oregon results had been posi-
tive. This could have resulted in agreement on 13
slides at most. The presence or absence of clinical
symptoms consistent with pertussis did not corre-
late with the results of any one of the readers.

The fact that the three readers using the tech-
niques that would ordinarily be employed in their
respective diagnostic laboratories agreed on only
50% of smears indicates that although direct immu-
nofluorescence may be useful for rapid presumptive
diagnosis it would be hazardous to use it alone as a
diagnostic method without confirmatory cultures.
Methods are now needed for development and field
evaluation of transport and isolation media. Pre-

liminary reports on the Jones-Kendrick charcoal media and the Oxoid charcoal media are encouraging, but neither has yet been compared to direct plating on BG media in a field situation (6,12).

Bacteriologic technique enabled us to document asymptomatic carriage of *B. pertussis* in the Atlanta outbreak. Lambert, Linnemann, and Field also identified small numbers of asymptomatic, culture-positive individuals during outbreaks of pertussis (5,13,14). Evaluation of the epidemiologic significance of asymptomatic culture-positive individuals would be of interest.

The interpretation of the surveillance data on the age distribution of reported pertussis cases (Table 3) is hampered by reliance on a clinical diagnosis of pertussis. There is almost certainly a bias toward reporting cases that are clinically typical, which would occur predominantly in younger children.

The CDC, in collaboration with state health departments and physicians throughout the country, is implementing more complete reporting of pertussis cases beginning in late 1978. Information to be collected will include method of diagnosis, morbidity, and mortality, in addition to the age, sex, and geographic location of cases. At the same time, states are being encouraged to confirm as many diagnoses as possible by means of culture. A more complete investigation of cases and household contacts will be encouraged during outbreaks of pertussis. The form to be used in investigation of these cases will permit the calculation of vaccine efficacy based on the occurrence of secondary cases in households classified by their vaccination status. Questions will also be asked regarding the use of antibiotics for prophylaxis in household contacts. Patterns of secondary spread of disease within the household should also be defined more clearly.

Through the utilization of appropriate diagnostic techniques and increased surveillance, it should be possible to estimate morbidity and mortality due to pertussis. We also should be able to confirm the efficacy of the currently used vaccines. This information must be available if we are to make rational policy decisions in the face of the current controversy over the risks and benefits of immunization against pertussis.

BIBLIOGRAPHY

1. Hebert, G. A.; Pittman, B. V.; McKinney R. M.; Cherry, W. B. The preparation and physicochemical characterization of fluorescent antibody reagents. Center for Disease Control, Atlanta, 1972.
2. Brooksaler, F.; Nelson, J. D. Pertussis: a reappraisal and report of 190 confirmed cases. Am. J. Dis. Child. 114:389–396; 1967.
3. Chalvardjian, N. The laboratory diagnosis of whooping cough by fluorescent antibody and by culture methods. Can. Med. Assoc. J. 95:263–266; 1966.
4. Holwerda, J.; Eldering, G. Culture and fluorescent antibody methods in diagnosis of whooping cough. J. Bacteriol. 86:449–551; 1963.
5. Field, L. H.; Parker, C. D. Pertussis outbreak in Austin and Travis County, Texas, 1975. J. Clin. Microbiol. 6: 154–160; 1977.
6. Jones, G. L.; Kendrick, P. L. Study of a blood-free medium for transport and growth of *Bordetella pertussis*. Health Lab. Sci. 6:40–45; 1969.
7. Baraff, L. J.; Wilkins, J.; Wehrle, P. F. The role of antibiotics, immunizations, and adenoviruses in pertussis. Pediatrics 61:224–230; 1978.
8. Miller, J. J.; Leach, C. W.; Saito, T. M.; Humber, J. B. Comparison of the nasopharyngeal swabs and the cough plate in the diagnosis of whooping cough and *Hemophilus pertussis* carriers. Am. J. Public Health 33:839–843; 1943.
9. Brooks, A. M.; Bradford, W. L.; Berry, G. P. The method of nasopharyngeal culture in the diagnosis of whooping cough. J.A.M.A. 120:883–885; 1943.
10. Bass, J. W.; Klenk, E. L.; Kotheimer, J. B.; Linnemann, C. C.; Smith, M. H. D.; Mitchell, T. A.; Cover, E. J.; Leiderman, D. S. Antimicrobial treatment of pertussis. J. Pediatr. 75:768–681; 1969.
11. Linnemann, C. C. Jr.; Partin, J. C.; Perlstein, P. H.; Englender, G. S. Pertussis: persistent problems. J. Pediatr. 85:589–591; 1974.
12. Regan, J.; Lowe, F. Enrichment medium for the isolation of *Bordetella*. J. Clin. Microbiol. 6:303–309; 1977.
13. Lambert, H. S. Epidemiology of a small pertussis outbreak in Kent County, Michigan. Public Health Rep. 80:365–369; 1965.
14. Linnemann, C. C. Jr.; Ramundo, N.; Perlstein, P. H.; Minton, S. D.; Englender, G. S.; McCormick, J. B.; Hayes, P. S. Use of pertussis vaccine in an epidemic involving hospital staff. Lancet 2:540–543; 1975.

Purification and Biological Properties of *Bordetella pertussis* Antigen Binding to Complement-Fixing Antibodies Induced During Whooping Cough

R. Winsnes

ABSTRACT

The chemical nature and some biological properties of the protein constituting the main antigen in the complement-fixation test for both postinfection and postvaccination sera have been studied. The antigen was fractionated from the supernatants of 3-day cultures of *Bordetella pertussis*, strain CN 5612/67 (Wellcome), by ultrafiltration and ammonium sulfate precipitation followed by methanol precipitation, gel filtration, and sodium dodecyl sulfate polyacrylamide gel electrophoresis to obtain a soluble antigen.

For human sera two- to threefold higher complement-fixing antibody titers were obtained when partly purified soluble antigen was used instead of bacterial whole cell antigen. The partly purified soluble antigen provoked high complement-fixing antibody titers in mice. It exerted no mouse-protective, leukocytosis-promoting factor, or histamine-sensitizing factor activities and no pronounced stimulating effect on human lymphocytes in vitro.

The methanol-precipitated, partly purified soluble antigen inhibited the bactericidal effect of human convalescent sera on *B. pertussis*.

Specific activity of the antigen from gel filtration in complement-fixation tests was more than 200 times greater than that of the culture supernatant.

INTRODUCTION

A seroepidemiological survey, including investigation of immunity to whooping cough, to be performed in Norway during 1978 and 1979, requires a simple and reliable method for screening sera for content of antibodies against *Bordetella pertussis*.

The nature of the immunity which develops as a result of infection or vaccination has not been clearly defined. Winter (1), in comparisons of convalescent sera of whooping cough patients, found the percentages of the patients who showed increases in titer were 62% for agglutinins, 14% for hemagglutinins, and 100% for protective activity.

Brighton et al. (2) suggested that a direct serum bactericidal test might give a more reliable estimate of the state of immunity of individuals than, for instance, agglutination reactions, since antibody and complement are necessary for phagocytosis. Holt (3) described a tissue culture technique using *B. pertussis* adherent to fibroblasts as a substrate for the phagocytic activity of polymorphonuclear leucocytes.

The appearance of specific complement-fixing antibody (CFA) in serum after infection with *B. pertussis*, first reported by Bordet and Gengou

(4, 5), has been confirmed by others [cited by Wilson and Miles (6)]. Evans and Maitland (7, 8) found that agglutination was more sensitive than the complement-fixation test (CFT) for detecting antibodies against *B. pertussis*. But other studies (9–12) indicate that the CFT is more reliable than the agglutination test for diagnostic purposes.

A Norwegian study of 50 children injected with one, two, and three doses of diphtheria-pertussis-tetanus vaccine showed that each new dose resulted in increasing CFA titers when whole cell *B. pertussis* was used as antigen. Serum specimens taken early and late during natural infection, at least 2 weeks apart, showed a fourfold or greater increase in CFA titers (Kjennerud, U., personal communication, 1978).

Our review suggested that although the mouse protection test used by Winter (1) was most reliable in determining immunity against *B. pertussis*, it is too laborious and expensive compared with CFT. We therefore decided to investigate whether development of CFA is followed by development of protective antibody. If so, the CFT would be suitable for analyses of postvaccination serum samples.

We isolated protein that provokes high levels of CFA against *B. pertussis* in mice after vaccina-

tion. In high dilutions, the protein reacts as an antigen in CFTs using human convalescent sera. The chemical, immunochemical, and biological properties of various fractions from the purification procedure are presented in this report.

MATERIALS AND METHODS

Preparation of Partly Purified Soluble Antigen

B. pertussis, strain CN 5612/67 (Wellcome), was cultivated in a modification (13) of the liquid medium first described by Cohen and Wheeler (14), using anion exchange resin. The 3-day culture supernatant was centrifuged at 5° C at 7,970 × g for 20 minutes in a Sorvall RC2–B centrifuge with rotor GSA to remove bacteria. The supernatant was concentrated sevenfold by ultrafiltration in Amicon TC20–1, using Diaflo ×M50 membranes with a general retentivity of proteins with molecular weights above 50,000. Thimerosal (0.1%) was added to the concentrate. One part of concentrate was precipitated by addition of three parts of a

saturated solution (37° C) of ammonium sulfate. After precipitation at 4° C for 16 hours and centrifugation, the sediment was dissolved in saline.

Preparation of Soluble Antigen

To remove lipids, carbohydrate, and protein impurities, the partly purified soluble antigen was precipitated with 40% methanol at 2° C. The precipitate was further purified by gel filtration at 11° C on Sephadex G–75 columns (88 × 1.5 cm). The gel was equilibrated and eluted with 20% formic acid or with 0.1 M Tris-HCl buffer, pH 9.8, containing 1 M NaCl, 0.5% 2–mercaptoethanol, and 0.1% thimerosal (Fig. 1). In the latter case the samples were dissolved by addition of NaOH to pH 11.0 immediately before gel filtration.

The fraction eluted from columns with 20% formic acid was further purified by preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The protein fraction with a molecular weight of about 39,000 is the soluble antigen.
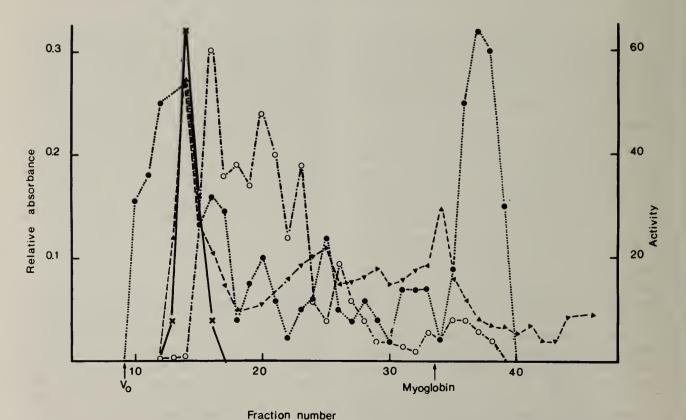

Figure 1. Gel infiltration of methanol fraction antigen on Sephadex G–75. Fractions of 3.5 ml were eluted with Tris-HCl buffer and tested for absorbance at 280 nm (●···●) and for antigen activity (×——×). Analyses of protein also were performed by the Bio-Rad protein assay (▶–▶–▶–▶). Carbohydrates were analyzed by the phenol-sulfuric acid assay (○·–·–·○).

The soluble antigen was eluted from slabs by slicing the unstained gel into 1-mm sections and extracting with the electrophoresis buffer overnight at 20° C. After removal of the gel slices, the eluate was dialyzed for 24 hours against 8 M urea to remove SDS and 48 hours against 0.9% saline at 11° C. Then CFTs, counterimmunoelectrophoresis, crossed immunoelectrophoresis, and protein determination were performed.

SDS-Polyacrylamide Gel Electrophoresis

Bio-Rad Model 220 was used for SDS–PAGE based on the methods of Laemmli (15) and Ames (16). The electrophoresis buffer was 0.025 M Tris, 0.19 M glycine, 0.1% SDS, pH 8.3. In some experiments the gels were prerun with a current of 13 mA for 10 minutes.

Lipopolysaccharides from *B. pertussis,* strain 18334, kindly provided by Professor A. C. Wardlaw, Glasgow, were also tested. For molecular weight estimations a reference mixture from Pharmacia Fine Chemicals, Sweden, was used.

Protein Analyses

Protein content was determined by a micro-Kjeldahl technique after TCA-precipitation and heat-coagulation (17) or by the Bio-Rad protein assay (18). Absorbance at 280 nm of column eluates was determined. Staining of polyacrylamide gels with Coomassie Blue was carried out as described by Ames (16) using the four stain steps recommended by Fairbanks et al. (19).

Amino Acid Analyses

Samples containing 0.2–1 mg protein were hydrolyzed under nitrogen in 6 N HCl for 24 hours. The automatic amino acid analyses were carried out as described by Sletten and Husby (20). Tryptophan was not determined.

Detection of Carbohydrates and Fatty Acids

The elution of carbohydrates from columns was monitored by the phenol-sulfuric acid assay (21). Polyacrylamide gels, previously stained for protein, were investigated for carbohydrate content by the periodic acid-Schiff procedure, used essentially as described by Fairbanks et al. (19).

Fatty acids were detected by gas chromatography after treatment of samples with HCl in anhydrous methanol, followed by trifluoroacetylation of the methanolysate (23).

Immunological Techniques

Counterimmunoelectrophoresis was carried out on glass plates coated with a 0.1 cm layer of 1% agarose (Agarose A-37 from L'Industrie Biologique Francaise) in a barbital/calcium lactate buffer, pH 8.6, $\mu = 0.065$. Sera containing antibody against *B. pertussis* were placed in a row of wells near the anode and various antigen-containing fractions were added to wells near the cathode. Electrophoresis was carried out for 20 minutes at 10 V/cm (Figs. 5,7).

Crossed immunoelectrophoresis was performed according to the method of Weeke (23), using the agarose gel and electrophoresis buffer described for counterimmunoelectrophoresis. The gel of the second dimension contained 11.0% pooled human convalescent serum or 0.8% rabbit immune serum. The field strength was 10 V/cm for 30 to 60 minutes in the first dimension and 3 V/cm for 17 hours in the second dimension. In some experiments the sample solution contained 0.1% SDS.

A micromethod was used for determination of CFA titer (24), using a barbital buffer with calcium and magnesium. In this study 2.5 doses of hemolytic complement were used. "Checkerboard" titrations were performed to find the optimal concentration of antigens (24). For titration of sera, three antigen units were used. In addition to the various fractions from the 3-day culture supernatant, *B. pertussis,* CN 5612/67 (5×10^9 bacteria/ml) and strain 3803/57 (Statens Seruminstitut, Copenhagen) (5×10^9 bacteria/ml) were used as antigens in CFTs. The whole bacterial antigens were harvested by centrifugation from 3-day liquid culture medium. After suspension in 0.9% saline containing thimerosal (0.1%), the bacterial antigens were incubated at 56° C for 30 minutes.

All titers in this study are expressed as reciprocals.

Serum Samples

The human immune serum pool, used in studies of antigen titer of fractions, in counterimmunoelectrophoresis, in crossed immunoelectrophoresis, and in bactericidal assays, consisted of sera from three convalescents from whooping cough.

Murine sera for studies of CFA development were obtained 15 days after a single intraperitoneal injection of 0.5 ml of the respective vaccines. Each vaccine was injected into 10 mice. One dose of vaccine A or vaccine B contained 313 μg protein corresponding to about 3×10^{10} bacteria/ml.

One dose of vaccine C or D contained 375 μg protein.

Two rabbits were immunized by intradermal injections of *B. pertussis* CN 5612/67 in Freund's Incomplete Adjuvant. The immunization schedule consisted of five injections, a 5-day interval, then two injections and a 2-week interval. Each animal received 10^{10} to 2×10^{10} bacteria/dose. Serum obtained 3 months after primary injection was used in these studies. CFA titer of this serum was 1,600.

Another rabbit was injected with the culture medium (13) using the same immunization schedule.

Mice

Inbred female mice of the NMRI/BOM strain were used. In leukocytosis-promoting factor (LPF) assays comparable results were obtained with mice of 4, 6, and 8 weeks of age. Mice used for hista-mine-sensitizing factor (HSF) assays and for producing immune sera were 7 weeks old.

Vaccines

For comparison in assays a plain vaccine containing 41 P.U./ml was made from *B. pertussis*, strain CN 5612/67 (Wellcome), cultivated on Bordet-Gengou agar plates for 3 days (vaccine A). Another plain vaccine was made from strain CN 5612/67 cultivated for 3 days in the modified medium of Cohen and Wheeler (vaccine B). The latter vaccine contained less than 0.47 P.U/ml.

Three vaccines were made from the 3-day culture supernatant of strain CN 5612/67. Vaccine E, an aluminum phosphate-adsorbed vaccine, was prepared from the 40% methanol fraction. Vaccine C, an adsorbed vaccine, and vaccine D, a plain vaccine, were prepared from the ammonium sulfate fraction of the XM50 concentrate (Table 1). Vaccines C, D, and E contained less than 0.47 P.U./ml. Adsorbed vaccines were prepared by dialyzing the fraction three times against a 2% solution of dibasic sodium phosphate for 2 hours, adding 10% aluminum chloride dropwise until pH 4.1 was reached, and immediately suspending the precipitate in phosphate-buffered saline, pH 7.2. Extreme pH values were avoided (25). Thimerosal (0.1%) was added to the vaccines, which were heated to 56° C for 30 minutes before use. Vaccine D (4.2 mg protein/ml) was lethal for mice before heat inactivation, but vaccine C (1.5 mg protein/ml) lacked this effect.

Table 1. Recovery of Antigen Activity Estimated by Complement-Fixation Tests Using A Pool of Human Convalescent Sera

Fraction	Recovery of Antigen [a] (%)	Specific Antigen Activity [b]
Culture supernatant	100	10
Concentrate from ultrafiltration	87	50
Partly purified soluble antigen [c]	135	320
Methanol-precipitated antigen [d]	108	360
Antigen eluted with 20% HCOOH [e]	50	400
Soluble antigen [f]	N.D. [g]	>100 [h]
Antigen eluted with Tris/HCl buffer containing 2–mercaptoethanol [e]	40	>2000 [h]

[a] Antigen titer × volume; [b] antigen titer/mg protein/ml; [c] precipitated with ammonium sulfate; [d] partly purified soluble antigen precipitated with 40% methanol; [e] from Sephadex G–75 columns; [f] from SDS–PAGE; [g] not determined; [h] too little protein for exact determination.

Lymphocyte Stimulation Studies

Partly purified soluble antigen from the 40% methanol fraction was tested for effect on human lymphocytes after extensive dialysis against 0.9% saline to remove possible traces of thimerosal. Four concentrations of protein were tested (200 μg/ml, 20 μg/ml, 2 μg/ml, and 0.2 μg/ml).

Peripheral human lymphocytes were isolated by the method of Bøyum (26). Triplicate cultures of 150 μl containing 1.5×10^5 lymphocytes/well were placed in Nunclon microtiter plates (Roskilde, Denmark) and incubated at 37° C in a humidified atmosphere of CO_2 in air for 2 or 4 days after addition of 10 μl sample solution. Purified phytohemagglutinin (PHA) (HA 16) (Wellcome Reagents Ltd.) was used as a control at a pretested optimal concentration.

Ten μl of a solution containing 0.7 μCi of ^3H-thymidine (Radiochemical Centre, Amersham, England) was then added to each well. The microtiter cultures were harvested and washed 24 hours later. ^3H content was determined in a liquid scintillation spectrometer (Packard, model A 2425).

The experiment was repeated twice for both 3- and 5-day incubation periods, using different lymphocyte donors.

Inhibition of Bactericidal Activity

The convalescent serum pool described was used as immune serum. Serum from a 23-year-old female who had never had clinical manifestations of natural pertussis infection was used as negative serum.

This serum was completely negative in CFTs at a dilution of 1:4.

Suspensions in 1% casamino acids of 2-day cultures from Bordet-Gengou medium of *B. pertussis*, strain CN 5612/67 were made to give a maximum of 300 colonies/plate of Bordet-Gengou medium in the bactericidal assay.

Inhibition of serum bactericidal activity by the 40% methanol fraction (24 μg protein/30 μl of saline/tube) was studied in two ways.

In the first experiment 30 μl of either 0.9% saline or methanol fraction in 0.9% saline was mixed with 30 μl of the appropriate immune serum dilution and incubated in a water bath at 37° C for 30 minutes prior to addition of 30 μl of 0.9% saline and 30 μl of bacterial suspension in 1% casamino acids. The mixture was incubated at 37° C for 30 minutes and 50 μl volumes were spread on the surface of Bordet-Gengou agar plates. The plates then were incubated in a moist chamber at 35° C for 3 days, after which colonies were counted. Controls contained 0.9% saline instead of serum antibody.

In the second experiment the immune serum was heat-inactivated at 56° C for 30 minutes before mixing with saline or methanol-fraction antigen, and 30 μl of undiluted negative serum was added instead of saline before final incubation.

Each experiment was run two times in duplicate.

Bioassays

Vaccines were assayed by the intracerebral mouse protection test (27,28), using *B. pertussis* 18323 as the challenge strain.

A Norwegian standard vaccine combined with vaccine D (134 μg protein of vaccine D/ml standard vaccine) was also tested for protective activity.

Ten unvaccinated mice were injected intraperitoneally with murine immune serum (0.2 ml/mouse) 16 hours before intracerebral challenge with strain 18323. The murine immune serum came from mice immunized with an ordinary vaccine (0.4 P.U./mouse). The vaccine consisted of *B. pertussis* strains CN 5612/67, 5611/70, and 5621/67 (Wellcome). The immune serum was drawn 15 days after intracerebral challenge of the mice with strain 18323 and was incubated for 30 minutes at 37° C before injection.

Another 10 mice were injected with adsorbed murine immune serum 16 hours before challenge in the mouse protection test. The immune serum was adsorbed, by incubation for 30 minutes at 37° C in a water bath, with methanol-fraction antigen (228 μg protein/ml). The antigen activity estimated by CFTs, was removed by filtration through a Millipore membrane filter (0.22 μm).

The CFA titers of the unadsorbed and the adsorbed serum were 256 and less than 10 respectively. Partly purified soluble antigen was used as antigen in these CFTs.

Vaccines A, B, C, and D passed the weight gain freedom from toxicity test (28).

Four fivefold dilutions of the respective vaccines were used in studies of HSF activity. Ten mice were used for each dilution point. The first dilution level received the following doses: vaccines A and B, 3×10^{10} bacteria; the International Standard for Pertussis vaccine, 10^{10} bacteria; vaccine D, 419 μg protein; and 105 μg protein of the plain vaccine 40% methanol fraction. The challenge dose consisted of histamine dihydrochloride (6 mg/mouse). The challenge was given 4 days after vaccination. All the injections were intraperitoneal.

LPF assays were carried out with the same vaccines and dilutions as in HSF tests. On the second, third, fourth, and fifth days after vaccination, total white blood cell counts were measured in a Coulter Counter, Model Z_B (Coulter Electronics Ltd., Harpenden Herts, England).

Assays of LPF activity of vaccine A (313 μg protein/0.5 ml) supplemented with methanol fraction (313 μg protein) were also performed.

RESULTS

Because of higher specific antigen activity, 3-day culture supernatants were found to be superior as starting material for fractionation to 2-day culture supernatants or extracted bacteria.

Recovery of antigen and specific antigen activity in the fractionation procedure is shown in Table 1. Specific activity of antigen from gel filtration was more than 200 times higher than that of the culture supernatant. Preliminary experiments with gels containing agarose, dextran, polystyrene, or hydroxy-apatite indicated a strong binding of antigen to the gels, so the loss of 60% of antigen by filtration on Sephadex G–75 (Table 1) was not surprising. The antigen activity was eluted shortly after the void volume (Fig. 1). Taking 70,000 as the inclusion limit, the molecular weight, calculated from the gel elution pattern, was about 39,000 (Fig. 1). The UV spectrum contained no peak. A strongly UV-absorbing fraction was separated from the protein containing antigen activity after addition of NaOH to pH 11.0 to the sample

solution prior to gel filtration (Fig. 1). The molecular weight calculated as for a protein was about 16,000. A shoulder was observed in the UV spectrum between 245 and 262 nm.

When the methanol fraction was dissolved in and eluted with 20% formic acid from Sephadex G–75 columns, the material included in the 16,000 dalton peak in Figure 1 presumably eluted together with the antigen activity. The amino acid composition of the antigen fraction eluted with 20% formic acid is given in Table 2. Glycine, alanine, asparagine, and glutamic acid were the predominant amino acids.

The methanol fraction gave a main protein band of about 78,000 daltons and a weak band of about 39,000 daltons when applied to the electrophoresis gel before the current was switched on (Fig. 2). When the sample was added after 10 minutes of electrophoresis, only the 39,000 dalton band appeared.

The Sephadex G–75 fraction was strongly positive for carbohydrates, evident from SDS–PAGE. Controls showed that most of the contaminating carbohydrates originated from the gel material. A major protein band with electrophoretic mobility corresponding to a molecular weight of about 39,000 appeared whether the sample was applied before or after the current was switched on (Figs. 2,3). When the electrophoretic conditions of Morse (29) were used, a corresponding major protein band was detected on the slab.

The degree of purification of soluble antigen in the concentrated ultrafiltrate of culture supernatant

Figure 2. SDS-polyacrylamide gel electrophoresis of references, methanol fraction antigen, the latter antigen incubated in 20% HCOOH at 11°C for 16 h, and the antigen fraction eluted with 20% HCOOH from Sephadex G–75 (from left to right). The gel was not prerun. Phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin were used as references.

is indicated in Figure 3. The soluble antigen did not stain for carbohydrate. Lipopolysaccharides could not be demonstrated on polyacrylamide gels stained for protein and carbohydrate. Gas chromatography of the methanol fraction showed no fatty acids; i.e., less than 0.1% of the fraction could consist of lipopolysaccharides.

Tables 3 and 4 illustrate "checkerboard" titration of methanol fraction antigen and of antigen eluted from columns with Tris-HCl buffer containing 2-mercaptoethanol (Fig. 1) respectively. In repeated tests the CFA titer of the human convalescent serum pool was slightly higher with antigen from Sephadex G–75. A more distinct endpoint was noticed for the methanol fraction antigen.

Counterimmunoelectrophoresis of the methanol fraction antigen against either rabbit or human immune serum formed two precipitation lines (Fig. 4). No lines were visible against serum of the rabbit immunized with the culture medium of B. pertussis. When a solution of the methanol fraction was adjusted to pH 11.0 with NaOH and dialyzed against 0.9% saline for 1 hour before counterimmunoelectrophoresis, two precipitation lines were also formed against rabbit serum, but only a weak, diffuse band appeared against human convalescent

Table 2. Amino Acids Detected After Acid Hydrolysis of the Soluble Antigen Eluted From Sephadex G–75 With 20% HCOOH

Amino Acids	Molar Proportions (Threonine = 1)
Cysteine	0.23
Asparagine	2.16
Threonine	1.00
Serine	0.97
Glutamic acid	2.12
Glycine	3.00
Alanine	3.01
Valine	1.50
Methionine	0.23
Isoleucine	0.80
Leucine	1.50
Phenylalanine	1.73
Histidine	0.27
Lysine	1.23 [a]
Arginine	0.27
Proline	trace

[a] The figure includes impurities.

Figure 3. SDS-polyacrylamide gel electrophoresis of concentrate after ultrafiltration of 3-day culture supernatant (left), references (middle), and antigen fraction eluted with 20% HCOOH from Sephadex G–75 (right). The gel was prerun for a short time before sample application. The same references as in Figure 2 were used.

serum (Fig. 4). The antigen eluted from columns with Tris-HCl buffer (Fig. 1) formed two lines against rabbit serum and one line against human convalescent serum. No line was visible against the ~16,000-dalton fraction from the Sephadex column.

Crossed immunoelectrophoresis of rabbit antiserum and partly purified soluble antigen formed several precipitation lines (Fig. 5). The soluble antigen gave a single line against the human convalescent serum pool (Fig. 6). However, it gave one broad or possibly two diffuse lines in counterimmunoelectrophoresis against rabbit immune serum (Fig. 7). The soluble antigen had high specific antigen activity in CFTs (Table 1). The CFA titer of the human convalescent serum pool was 160 using the soluble antigen.

Retention of activity of the soluble antigen after 5 minutes in a boiling water bath, prior to SDS–PAGE, was consistent with preliminary heat stability studies which showed that one-eighth of the specific activity of partly purified soluble antigen still remained after heating in a water bath at 94° C for 30 minutes.

Table 3. Titration of Antigen and Standard Antiserum

Reciprocal Dilutions of Methanol-Precipitated Partly Purified Antigen	Reciprocal Dilutions of Antiserum						Antigen Control
	10	20	40	80	160	320	
4	4	4	4	3	2	0	0
8	4	4	4	3	2	0	0
32	4	4	4	3	2	0	0
64	4	4	3	2	0	0	0
128	4	3	2	1	0	0	0
256	4	2	1	0	0	0	0
512	3	1	0	0	0	0	0

Table 4. Titration of Antigen and Standard Antiserum

Reciprocal Dilutions of Antigen From Sephadex G–75[a]	Reciprocal Dilutions of Antiserum								Antigen Control
	8	16	32	64	128	256	512	1024	
1	4	N.T.[b]	N.T.	N.T.	2	2	N.T.	N.T.	0
2	4	4	4	4	3	2	2	1	0
4	4	4	4	4	3	2	2	1	0
8	4	4	4	4	3	2	2	1	0
16	4	4	4	3	2	1	0	0	0
32	4	3	2	1	0	0	0	0	0
64	4	2	1	0	0	0	0	0	0
128	2	1	0	0	0	0	0	0	0

[a] Partly purified soluble antigen precipitated with 40% methanol and eluted with Tris-HCl buffer containing 2-mercaptoethanol from Sephadex G–75 columns, [b] not tested.
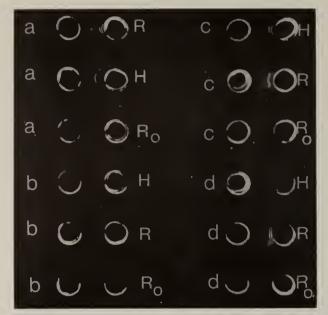
Figure 4. Counterimmunoelectrophoresis against human convalescent serum (H), rabbit immune serum (R), and rabbit control serum (R_o) of methanol fraction antigen (c), the latter exposed to pH 11.0 (d), the antigen fraction eluted with Tris-HCl buffer from Sephadex G–75 (a), and the strongly UV-absorbing fraction from the same column (b). Anode to the right.

It was also very stable against acids and bases; incubation at 20° C for 24 hours at pH 1.0 or 11.0 resulted in no decrease of specific antigen activity.

Partly purified soluble antigen adsorbed on aluminum phosphate (<0.47 P.U./ml, vaccine C), provoked a high titer of CFA against *B. pertussis* 3803/57 whole cell antigen (Table 5). A tenfold higher titer was found when partly purified solu-
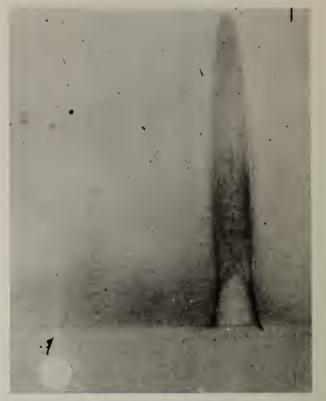
Figure 6. Crossed immunoelectrophoresis against human convalescent serum of the soluble antigen. Anode to the right and top.

ble antigen was used in CFTs. A plain bacterial whole cell vaccine (A) containing 41 P.U./ml induced CFA of comparatively low titer in mice. It provoked an even lower titer of CFA than another whole cell vaccine (B) containing less than 0.47 P.U./ml (Table 5).

Figure 5. Crossed immunoelectrophoresis against rabbit immune serum of partly purified soluble antigen. Anode to the right and top.
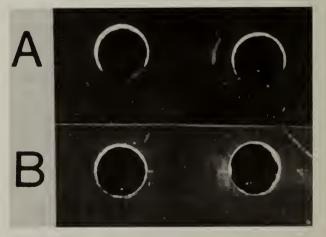

Figure 7. Counterimmunoelectrophoresis of the methanol fraction antigen (A) and the soluble antigen (B) against rabbit immune serum. Anode to the right.

Table 5. Content of Complement-Fixing Antibodies Against *B. pertussis* in Sera of Mice Injected With Various Pertussis Vaccines

| Vaccine Administered [a] | Protective Units/ml [b] | CFA [c] Titers of Murine Sera Using: | |
		Whole cell antigen [d]	Partly purified soluble antigen [e]
A [f]	41	60	640
B [g]	< 0.47	240	960
C [h]	< 0.47	1 280	12 800
E [i]	< 0.47	5	160

[a] From *B. pertussis* CN 5612/67; [b] calculated by the Wilson-Worcester method for determining the median effective dose of pertussis vaccine; [c] complement-fixing antibody; [d] *B. pertussis* no. 3803/57 antigen (90 μg protein/ml); [e] containing 4.8 μg protein/ml; [f] cultivated on Bordet-Gengou agar plates; [g] cultivated in modified Cohen and Wheeler's medium; [h] adsorbed partly purified soluble antigen; [i] adsorbed methanol-precipitated ultrafiltrate.

The ability of the methanol fraction antigen to bind with CFA of human convalescent serum was also verified by bactericidal assays (Table 6). A serum dilution of 1:128 left no survivor when the convalescent serum was incubated with saline before addition of *B. pertussis*. After the serum had been adsorbed with methanol fraction antigen, the highest serum dilution leaving no survivor was 1:8.

When convalescent serum was used as a source of both CFA and complement, the highest serum dilution giving no survivor was 1:8 for unadsorbed human convalescent serum. Even undiluted serum adsorbed with methanol fraction antigen gave 2% survivors.

Neither the partly purified soluble antigen nor the methanol fraction exerted any noticeable protective effect in the intracerebral challenge test (Table 5). Addition of partly purified soluble antigen had no effect on the mouse protective activity of the Norwegian standard vaccine.

Experiments were performed to see if the methanol fraction antigen could remove the protective activity of murine serum by adsorption. The serum pool used was drawn from mice 4 weeks after initial vaccination. Unfortunately, the serum had

little protective activity when given to unvaccinated mice 16 hours before intracerebral challenge. However, the 10 mice given the adsorbed serum died 1 day before the 10 mice given unadsorbed serum.

In vitro lymphocyte stimulation studies revealed borderline activity with a ratio of three of the partly purified soluble antigen. PHA had 30 times greater effect.

The results of the vaccines A and B and the international standard for pertussis vaccine in LPF and HSF assays were consistent with the literature (31,32). No LPF or HSF activities were discovered for the partly purified soluble antigen *or* the methanol fraction antigen. Neither suppressed or increased the LPF activity of vaccine A.

CFTs, repeated twice, of 20 postinfection and 10 postvaccination human sera gave a two- to threefold higher titer for the respective sera when partly purified soluble antigen was used instead of bacterial whole cell antigen of strain CN 5612/67.

DISCUSSION

The molecular weight of the soluble antigen, estimated before the importance of a short pre-electrophoresis was realized, was about 78,000. After pre-electrophoresis the molecular weight was about 39,000. Residual ammonium persulfate in the gel probably neutralized the reducing effect of 2-mercaptoethanol in the sample solution, causing the formation of disulfide linkages. Consistent with this, cysteine was detected (Table 2) in the antigen fraction from gel filtration.

The soluble antigen did not stain for carbohydrates on polyacrylamide gels, although minor quantities of carbohydrates might have been present. As the UV spectrum of the antigen fraction eluted from Sephadex G–75 (Fig. 1) contained no peak, the protein may have been contaminated. Adjusting the sample solution to pH 11.0 before gel filtration resulted in separation of a strongly UV-absorbing fraction. The UV spectrum of the fraction, which eluted after myoglobin, was not typical for nucleic acids and will be studied further.

Table 6. Bactericidal Antibody Titer of Human Convalescent Serum Pool Against *B. pertussis* CN 5612/67, Expressed as Reciprocal of the Highest Dilution Giving 0% Survivors

Primary Incubation	Added Before Final Incubation	Antibody Titer
Saline + immune serum	Negative serum + *B. pertussis*	>128
Antigen [a] + " "	" " "	8

[a] Partly purified soluble antigen precipitated with 40% methanol

As the least alkali-resistant ribonucleic acids are cleaved at pH 11, the antigen might be a nucleoprotein originating from the ribosomes.

Experiments indicating that extracted bacteria were less suitable as antigen in CFTs are consistent with speculation that the antigen is not derived from the bacterial surface. Furthermore, higher CFA titer of both human postinfection and postvaccination sera and of murine postvaccination sera were obtained using partly purified soluble antigen instead of unextracted bacterial whole cell antigen. Therefore the compound possessing the main antigen activity in CFTs may not be fully exposed on the bacterial surface. The antigen, capable of inducing high titers of CFA in mice and of binding with CFA in human convalescent sera, might be derived from lysed bacteria and partly adsorbed to the bacterial surface in the liquid culture.

Still higher titers were found using the antigen from Sephadex G–75 columns (Fig. 1) in CFTs. This may indicate removal of inhibitors during the purification procedure and uncovering of antigenic determinants against which CFA has been produced in vivo.

The methanol fraction antigen adsorbed the bactericidal activity of human convalescent serum, indicating that the methanol treatment was harmless. The need for serum factors other than antibodies for bactericidal activity was clearly demonstrated by the low bactericidal effect obtained when diluted serum was the only source of complement. Corresponding results have been reported by others (2).

The fraction from gel filtration (Fig. 1) containing antigen activity in CFTs formed two precipitation lines against rabbit immune serum in counterimmunoelectrophoresis. We have not determined whether the formation of two precipitation lines is due to participation of both IgM and IgG antibodies or to the presence of two antigens. However, in crossed immunoelectrophoresis against human convalescent serum only one precipitation line appeared.

It may be argued that the bacterial whole cell vaccines should have been aluminum phosphate-adsorbed for a fair trial of CFA formation in mice. An important point is that a vaccine containing over 80 times more P.U./ml than another whole cell vaccine was a poorer inducer of CFA.

It is not known if the antigen fraction separated from the strongly UV-light-absorbing material on columns (Fig. 1) can induce CFA in animals. Amino acid analyses after further purification of the antigen fraction (Fig. 1) should be performed.

The murine serum used to protect mice passively before intracerebral challenge had minor protective activity. This was not unexpected, since Wardlaw (32) found that the challenge injection itself did not appear to act as a booster stimulus for antibody production in once-vaccinated mice. The murine serum might have been drawn too early, for as Pittman (12) has pointed out, a striking rise in human serum protective activity appears 5 weeks after onset of whooping cough. This experiment will be repeated when protective serum is available.

Partly purified soluble antigen had no protective activity in mice by the intracerebral challenge test and no activity in LPF or HSF assays. The low stimulating effect on human lymphocytes in vivo may be consistent with a secondary immune response.

In humans *B. pertussis* is a noninvasive organism, with a marked tendency to attach to the ciliated respiratory epithelium (33). An antigen that induces formation of CFA is therefore of no obvious importance in resistance against infection. If the antigen is localized in the cytoplasm, it may escape the human defense mechanisms during the initial stage of infection. If toxic agents causing clinical manifestations penetrate the body, as they must during whooping cough, CFA against such agents might be important. But antibodies against toxic substances are not detectable when whole cell bacterial antigen or culture supernatant is used in CFTs.

From these results the soluble antigen seems to be worthless for estimation of protective antibody level after vaccination. If different strains of *B. pertussis* have the soluble antigen in common, it may be of value in diagnosis of whooping cough.

ACKNOWLEDGMENT

REFERENCES

1. Winter, J. L. Development of antibodies in children convalescent from whooping cough. Proc. Soc. Exp. Biol. Med. 83:866–870: 1953.

2. Brighton, W. D.; Lampard, J.; Sheffield, F.; Perkins, F. T. Variation of killing power of human sera against *Bordetella pertussis*. Clin. Exp. Immunol. 5:541–548; 1969.

3. Holt, L. B. A new method for the measurement of the opsonin titres of antisera to *Bordetella pertussis*. J. Biol. Standard. 3:139–144; 1975.

4. Bordet, J.; Gengou, O. Le microbe de la coqueluche. Ann. Inst. Pasteur 20:731–741; 1906.

5. Bordet, J.; Gengou, O. Note complementaire sur le microbe de la coqueluche. Ann. Inst. Pasteur 21:720–726; 1907.

6. Wilson, G. S.; Miles, A. A. Topley and Wilson's principles of bacteriology and immunity, 5th edition, vol. 2. London: Arnold; 1964: 2008.

7. Evans, D. G.; Maitland, H. B. The failure of whooping cough sera to neutralise pertussis toxin. J. Path. Bact. 48:465–467; 1939.

8. Evans, D. G.; Maitland, H. B. Agglutination as a diagnostic test for whooping cough. J. Pathol. Bacteriol. 48:468–470; 1939.

9. Donald, A. B. The diagnosis of whooping-cough Br. Med. J. 2:613–618: 1938.

10. Cruickshank, R. Diagnosis of whooping cough: comparison of serological tests with isolation of *Bordetella pertussis*. Br. Med. J. 4:637–639; 1970.

11. Bradstreet, C. M. P.; Tannahill, A. J.; Edwards, J. M. B. Detection of *Bordetella pertussis* antibodies in human sera by complement-fixation and immunofluorescence. J. Hyg. (Camb.) 70:75–83; 1972.

12. Pittman, M. Protective activity of whooping-cough convalescent serum and serum-IgA level in mice infected with *Bordetella pertussis*. Lancet II:156; 1976.

13. Pusztai, Z.; Joó, I; Kiss, I. Some observations on the growth of *Bordetella pertussis* in liquid shaken cultures. Pathol. Microbiol. (Basel) 23:193–200; 1960.

14. Cohen, S. M.; Wheeler, M. V. Pertussis vaccine prepared with phase I cultures grown in fluid medium. Am. J. Public Health. 36:371–373; 1946.

15. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685; 1970.

16. Ames, G. F.–L. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. J. Biol. Chem. 249:634–644; 1974.

17. Pope, C. G.; Linggood, F. V. Purification of diphtheria toxoid. Br. J. Exp. Pathol. 20:297–304; 1939.

18. Bio-Rad Laboratories, Technical Bulletin no. 1051.

19. Fairbanks, G.; Steck, T. L.; Wallach, D. F. H. Electrophoretic analysis of the major polypetides of the human erythrocyte membrane. Biochemistry 10:2606–2617; 1971.

20. Sletten, K.; Husby, G. The complete amino-acid sequence of amyloid fibril protein AS in rheumatoid arthritis. Eur. J. Biochem. 41:117–125; 1974.

21. Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. C. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350–356; 1956.

22. Jantzen, E.; Bryn, K.; Bøvre, K. Gas chromatography of bacterial whole cell methanolysates. IV. A procedure for fractionation and identification of fatty acids and monosaccharides of cellular structures. Acta Pathol. Microbiol. Scand. [B] 82:753–766; 1974.

23. Weeke, B. Crossed immunoelectrophoresis. Scand. J. Immunol. 2, Suppl. 1:47; 1973.

24. Lennette, E. H. Complement fixation tests. Lennette, E. H.; Schmidt, N. J. eds. Diagnostic procedures for viral and rickettsial infections, 4th edition, New York: American Public Health Association, Inc.; 1969:52–56.

25. Joó, I; Pusztai, Z.; Juhász, V. P. Histamine-sensitizing activity of various pertussis vaccines. Z. Immunitätsforsch. Exp. Ther. 121:143–157; 1961.

26. Bøyum, A. Separation of leucocytes from blood and bone marrow. Scand. J. Clin. Lab. Invest. 21:Suppl. 97; 1968.

27. Kendrick, P. L.; Eldering, G.; Dixon, M. K.; Misner, J. Mouse protection tests in the study of pertussis vaccine. Am. J. Public Health 37:803–810; 1947.

28. W.H.O. Expert Committee on Biological Standardization. Proposed requirements for diphtheria toxoid, pertussis vaccine, tetanus toxoid and combined vaccines. (Requirements for Biological Substances Nos. 8/10.); No. 1182; 1978.

29. Morse, S. I. Lymphocytosis-promoting factor of *Bordetella pertussis*: isolation, characterization, and biological activity. J. Infect. Dis. 136:234–238; 1977.

30. Sato, Y.; Arai, H.; Suzuki, K. Leukocytosis-promoting factor of *Bordetella pertussis*. III. Its identity with protective antigen. Infec. Immun. 9:801–810; 1974.

31. Pittman, M. Determination of the histamine sensitizing unitage of pertussis vaccine. J. Biol. Stand. 3:185–191; 1975.

32. Wardlaw, A. C.; Jakus, C. M. Intracerebral mouse protection test for pertussis vaccine. I. Apparent absence of humoral protective antibody under the usual test conditions. Can. J. Microbiol. 14:989–997; 1968.

33. Collier, A. M.; Peterson, L. P.; Baseman, J. B. Pathogenesis of infection with *Bordetella pertussis* in hamster tracheal organ culture. J. Infect. Dis. 136:196–203; 1977.

Seroresponse to Pertussis Vaccine

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ABSTRACT

In Finland, almost all isolated pertussis strains are serotype 1.2. A widely used adsorbed DTP vaccine contained five opacity units (OU) of *B. pertussis* strain 18530 per dose. Twenty-one infants who were immunized three times with this vaccine had no agglutinin 2 (titer <10) 1–5 months after the third injection, although 81% had agglutinin 3 (titer ≥ 10). The results were compared to a second group of 27 infants who were immunized according to the same program with a new DTP vaccine containing 2.5 OU of strain 18530 and 2.5 OU of strain 1772 per dose. Two months after the third injection, 55% had agglutinin 2 and 75% had type 3 agglutinins. The direct tube agglutination test shows that both strain 18530 and 1772 are of serotype 1.2.3. Agglutinin production test in rabbits revealed the low immunogenicity of agglutinogen 2 compared to other agglutinogens in vaccine containing strain 18530.

An enzyme-linked immunosorbent assay (ELISA) for measuring pertussis-specific antibodies of human immunoglobulin classes G and M is described. Formalin inactivated *B. pertussis* cells, adsorbed on polystyrene tubes, functioned as a solid-phase antigen. Pertussis-specific antibodies in the sera of children vaccinated with the DTP vaccine containing strains 18530 and 1772 were also determined by ELISA. No clear correlation to agglutination results was found. Seroconversion rate, defined as a twofold or greater rise in antipertussis IgG or agglutinin titer, was 97% according to ELISA and 79% according to the less sensitive agglutination test in blood samples taken 2 months after the third DTP injection. Preimmunization sera contained very low levels of pertussis antibodies. The mean antipertussis IgG value diminished significantly in sera taken 2 months compared to sera taken 12 months after the third DTP injection. This shows the need for a DTP booster, which in this study raised the IgG level to a mean A_{400}-value of 0.40 ± 0.15.

INTRODUCTION

According to official statistics, the incidence of whooping cough in Finland has been less than 10 cases per 100,000 population during the last 10 years. However, small local epidemics have occurred, especially among schoolchildren aged 7–12 years who have probably had no natural infections of pertussis and whose immunity due to vaccination has waned. Cases of pertussis at school age or later are often so mild that they are not always diagnosed as pertussis and reported. Epidemiologically, these cases are important as they evidently form a reservoir of whooping cough in a vaccinated society.

When the vaccination campaign against pertussis began in 1952–53, the incidence of the disease was over 500 per 100,000. According to the recommended basic immunization program, infants are vaccinated at the age of 3, 4, and 5 months with DTP vaccine. Approximately 90% of the children receive three doses of DTP vaccine. DTP booster immunization is recommended 1 year after the third dose but is usually given later, at the ages of 3 to 5 years or, in many cases, omitted.

In the seventies, practically all the pertussis vaccine used was aluminum hydroxyphosphate gel-adsorbed diphtheria and tetanus toxoid and pertussis vaccine, manufactured by the Central Public Health Laboratory in Helsinki. The composition of this vaccine has not changed since 1958: 5 opacity units (OU) of formalin inactivated *B. pertussis* strain 18530 per human dose. This strain is serotype 1.2.3 with agglutinogen 2 being weaker than the other main agglutinogens.

In 1974 we began serotyping isolated strains and found that half the strains were serotype 1.2 (Table 1). The following year, 75% of the strains were type 1.2. Since 1976, we have examined most of the notified cases and have found that 200 of 203 isolated strains were serotype 1.2. In view of this situation, we substituted part of the pertussis cells in the DTP vaccine with *B. pertussis* strain 1772, which contains agglutinogens 1,2, and 3. The total cell number per dose was not increased, and the potency of the vaccine as measured by mouse protection test did not change significantly. We followed the seroresponse to this vaccine in rabbits and children

Table 1. Serotypes of the *B. pertussis* Strains Isolated in the Central Public Health Laboratory, Helsinki

Year	Number of Isolations	Serotypes			
		1	1.2	1.3	1.2.3
1974	12	0	6	3	2
1975	30	0	22	5	2
1976	70	1	69	0	0
1977	104	1	102	0	1
1978 (first half)	29	0	29	0	0

using agglutination techniques and enzymes immunoassay. The results are presented in this paper.

MATERIALS AND METHODS

Cultures

Strains 353Z and 3747 were supplied by Dr. T. E. Roy, The Hospital for Sick Children, Toronto; strains GL353, H36, and 360E by Dr. N. W. Preston, University of Manchester; and NCTC 10905–NCTC 10910 by the Central Public Health Laboratory, London. Strain 18530 was a gift from Dr. G. Eldering, and strain 1772 originates from Dr. L. B. Holt.

Isolation of Strains

Perorally taken nasopharyngeal swabs were plated on Diamidine-Penicillin-Fluoride agar (1) manufactured by Orion Diagnostica, Helsinki. After 2–7 days growth at 37° C, suspected colonies were cultured on Cohen-Wheeler agar (2), which contained 20% horse blood, and identified (3). A pure culture was cultivated for 1–2 days before serotyping and lyophilization.

Serotyping of Strains

Monospecific sera for agglutinogen 1, 2, and 3 were prepared by the methods of Preston (4) and Chalvardjian (5). For agglutinin 1, a rabbit was immunized with strain 353Z and high titer serum was adsorbed with an autoclaved homologous strain and with *B. parapertussis*. For agglutinin 2, the serum of a rabbit immunized with strain 3747 was adsorbed with strain H36 and *B. parapertussis*. Agglutinin 3 was prepared by immunizing a rabbit with strain H36 and adsorbing the serum with GL353, 360E, and *B. parapertussis*.

The typing sera were diluted to a mean agglutination titer of 160. Agglutinations were done in tubes using a twofold dilution series of sera. The same volume of *B. pertussis* suspension with 6 OU/ml was added to each tube, and the tubes were incubated at 37° C for 2 hours, then left overnight at room temperature. The endpoint was a definite agglutination read with the naked eye.

The reliability of the typing sera and the uniformity of the results with those of other laboratories were checked by comparing our sera with the typing sera kindly provided by Dr. Nagel (Rijks-Instituut voor de Volksgezondheid, Netherlands) and Dr. Zakharova (WHO Reference and Research Center on *B. pertussis*, Gamaleya Institute, Moscow) and the typing sera prepared by the method of Bronne-Shanbury et al. (6)(Table 2).

The results are not contradictory when it is assumed that a strain has a certain agglutinogen if the titer against the homologous typing serum is at least one-quarter of the maximum titer of the serum.

Estimation of Agglutinins

Major agglutinins 1,2, and 3 in sera of children or immunized rabbits were determined by the method of Preston et al. (7). Agglutinin 1 was titrated with GL353. After adsorption with the same strain, agglutinins 2 and 3 were titrated with strains 360E and H36, respectively. Similar results were obtained by the method of Dolby and Stephens (8).

Vaccines

In vaccine production, *B. pertussis* strains 18530 and 1772 were cultivated for 2 days at 35° C on modified Cohen-Wheeler agar containing charcoal (9). After inactivation with 0.1% formalin for 3 days at 4° C, the cells were washed and suspended in a buffered thimerosal solution.

Both DTP and pertussis vaccine contained 10 OU of cells per ml, adsorbed in aluminium hydroxyphosphate gel. The mean potency assayed by mouse protection test was at least 4 IU per single human dose equal to 0.5 ml.

Enzyme-Linked Immunosorbent Assay, ELISA

Pertussis-specific immunoglobulin G and M antibodies in human sera were detected by the ELISA method described by Engvall and Perlmann (10). Polystyrene tubes (Mekalasi, Helsinki) were coated with formalin inactivated *B. pertussis* cells in phosphate buffered saline solution, pH 7.4 (PBS). One ml of a pertussis cell suspension of 60 opacity units was rotated in the tubes for 6 hours. Coating and incubations were done in a rotatory shaker (200 rpm) at 37° C. The cells could only be used for

coating twice. The second coating with 0.5% normal human serum albumin (HSA) increased the precision of the assay. Serum dilutions in PBS containing 0.2% HSA were incubated in the coated and washed tubes for 3 hours to obtain the maximum response for a high titer serum. Alkaline phosphatase conjugated, immunopurified, heavy chain specific, antihuman immunoglobulin G or antihuman immunoglobulin M (Orion Diagnostica, Helsinki) in a dilution of 1 to 500 in PBS with 0.2% HSA were added to the further washed tubes and incubated overnight. This dilution of the conjugates was chosen because the ratio between the results of a sample and the blank was high.

The bound phosphatase was assayed with p-nitrophenol (Sigma 104) as a substrate and a 30-minute reaction time at 30° C. The results were expressed as the absorbance at 400 nm (A_{400}) minus the A_{400} of the blank, which was handled in the same way but without the sample. All the samples were analyzed as triplicates. In IgG assay, the day-to-day variation was corrected by using the dilution of 1 to 5000 of the standard sample (Tussoglobin, lot 127 by Behringwerke). The coefficient of variation for the IgG assay of 1:2000 dilution of a patient serum (LET) tested 20 times was 5%. The great sensitivity of ELISA compared to agglutination is seen in Figure 1. Agglutination titers of Tussoglobin and LET serum were 800 and 1,280 respectively. The antipertussis IgG value of LET serum in 1:2000 dilution was 1.03.

RESULTS

Antigenic properties of the vaccine strains 18530 and 1772 were compared by direct agglutination in tube series (Table 2). Although no drastic difference was found, agglutinogen 2 in strain 18530 was
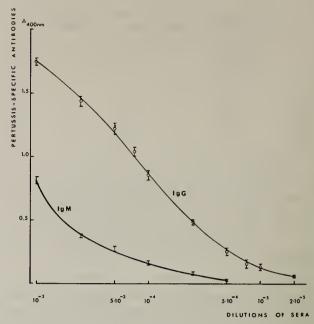

Figure 1. Titration curves of ELISA for antipertussis immunoglobulins G and M. Tussoglobin (lot 127) dilutions were analyzed for IgG and one patient serum (LET) for IgM. The mean values ± standard deviations calculated after subtracting the A_{400} of the blank from A_{400} of the samples are shown.

always lower than in strain 1772. A cultivation time of more than 2 days or a storage period of more than 2 years will diminish the amount of agglutinogen 2, especially in strain 18530.

Immunogenic properties of strains 18530 and 1772 were studied by their ability to produce major agglutinins in rabbits. The animals were immunized with DTP vaccines that contained different proportions of the two strains. The rabbit dose, 20 OU of pertussis cells, corresponded to the total dose for children.

As shown in Figure 2, strain 18530 has a strong agglutinogen 3 but very weak agglutinogen 2. Seven

Table 2. Comparison of the Agglutinogen Content of *B. pertussis* Strains Using Different Typing Sera. Titers are expressed as reciprocals of the highest final serum dilutions in which definite agglutination was observed.

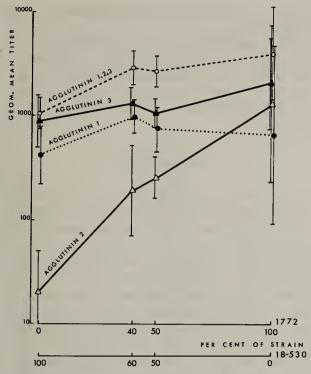| Strains	Serotypes											
	WHO Sera			R.I.V. sera			Sera type Preston (4)			Sera type Bronne-Shanbury (6)		
	1	2	3	1	2	3	1	2	3	1	2	3
87 SBL	80	<10	≥640	320	<40	640	40	10	160	1280	40	640
Po KTL	160	160	<20	640	320	<40	160	320	<10	640	160	<20
1772	80	80	320	≥320	160	≥320	80	160	80	640	160	320
18 530	20	40	≥640	160	80	≥320	80	80	160	640	20	320
G1-353	40	<10	<20				40	<5	<5	320	20	<20
360-E	80	160	<10				80	160	<10	320	320	<20
H-36	80	<10	160				80	<10	160	1280	<10	320

None of these childen had pertussis agglutinins before immunization. We analyzed agglutinins of 12 children 1 year after the third injection, and 6 of them had a titer ≥ 20 against vaccine strains.

The immunological response to the DTP vaccine that contained 2.5 OU of strain 18530 and 2.5 OU of strain 1772 was also studied by adapting the ELISA technique to measure pertussis-specific IgG and IgM antibodies. Formalin-inactivated pertussis cells adsorbed on the polystyrene tube functioned as antigen. In preliminary experiments, no difference was observed whether strain 18530 or 1772 was used as the antigen. The results are presented in Figure 3 and in Table 5, which also shows agglutination titers against strain 1772, including the corresponding titers of vaccinated children from Table 4. No clear correlation between the agglutination titers and A_{400} values for pertussis-specific IgG or IgM was found. Nor was there a correlation between monospecific agglutinins and ELISA results, as only sera with high titers have agglutinins 1,2, and 3.

If we define seroconversion as a twofold or greater rise in A_{400} or titer compared to preimmunization serum, then the seroconversion rate was 97% 2 months after the basic immunization, based on IgG values of ELISA, and 79% according to the less sensitive agglutination method (Table 5). Twelve months after the basic immunization, 3 of 20 children analyzed were not seroconverted, and the diminution of the mean value of antipertussis IgG was highly significant (Fig. 3.) One DTP booster increased the mean value to the same level as that after the basic immunization. Three infants whose pertussis antibodies were at the preimmu-

nization level before the booster injection were seroconverted.

The mean values of antipertussis IgM were very low in preimmunization sera $A_{400} = 0.02$ and in sera taken 2 and 12 months after the basic immunization $A_{400} = 0.06$. Following a booster injection, the mean value was significantly higher: 0.125 ± 0.07 SD.

DISCUSSION

In Finland, nearly all *B. pertussis* strains isolated during the last 3 years were serotype 1.2 (Table 1). As far as we know, this is the first report of the prevalence of this type in a vaccinated community. The strain Po KTL in Table 2 represents one typical isolation. Four different typing sera confirmed the lack of agglutinogen 3 in this strain, at least when our technique was used. When comparing serotyping results of different laboratories, one should keep in mind that by using high titer sera in slide agglutination, even residues of agglutinogens may give a positive reaction. As shown in Table 3, vaccinated children lack type 2 antibodies. The present situation agrees with Preston's suggestion (11) that the use of pertussis vaccines lacking a major agglutinogen would selectively proliferate strains containing the missing antigen. Unfortunately, we could not find older *B. pertussis* isolations to see if vaccination has changed the serotype, as has been observed in some communities (cf. 6, 11, 12).

Even a small difference in direct agglutination could, in some strains, mean a great difference in the ability to form agglutinins. Strains 18530 and 1772 have a difference of only about one dilution in agglutinogen 2 content (Table 2), but strain 18530 produced very little type 2 antibodies in rabbits (Fig. 2) and no measurable amounts of agglutinin 2 in infants (Table 3), whereas strain 1772 produced type 2 antibodies in both cases (Fig. 2 and Table 4). The direct agglutination technique did not reveal the low immunogenicity of agglutinogen 2 of strain 18530. The agglutinin production test in rabbits proved to be a more reliable method for determining the agglutinogen content of a pertussis vaccine.

Preston's group (7, 13) compared the response of four vaccines in rabbits and children and found that specific agglutinin production of a vaccine in rabbits predicted the agglutinin response in children.
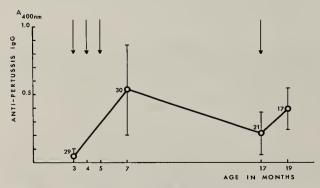

Figure 3. Immunological response to adsorbed DTP vaccine (lot 76327) measured with ELISA. Four injections were given to a group of 30 infants at times indicated by arrows. The number of analyzed blood samples and the mean value ± standard deviation of antipertussis IgG are shown. Individual assay results are presented in Table 5.

Table 5. Antibody Response of 30 Infants to Basic Immunization and Booster with Adsorbed DTP Vaccine Lot 76327. Three injections were given at 1-month intervals starting at the age of 3 months, the booster injection being given 12 months after the third injection. Pertussis specific IgG and IgM antibodies analyzed with ELISA using strain 1772 as antigen are expressed as corrected A_{400}-values (see Materials and Methods). The samples were diluted 1:500 before ELISA. The same antigen was used in the tube agglutination series. All infants had an agglutination titer less than 20 before vaccination.

Before vaccination		2 mos after 3rd injection			12 mos after 3rd injection		2 mos after booster	
IgG	IgM	IgG	IgM	Agglut.	IgG	IgM	IgG	IgM
0.01		0.22		80				
0.08		0.17		<20				
0.02		0.61		320				
0.01		0.75		160	0.24			
0.02	0.01	0.68	0.03	320	0.24	0.04	0.44	0.13
0.01		0.91		320	0.58			
0.04	0	0.90	0.21	320	0.27	0.01	0.38	0.19
0.03	0.02	0.38	0.04	320	0.08	0.05		
0.08	0.01	0.62		640	0.24	0.11	0.31	0.11
0	0	0.34	0.04	<20	0.09	0.03		
0.02	0.08	0.30		640	0.27	0.12	0.30	0.23
0.03		0.08		80	0.03		0.48	
0.03	0.01	0.23	0.03	320	0.10	0.03	0.26	0.27
0.01		0.54		640				
0.18		0.87		80	0.20		0.55	
0.03	0.02	0.30	0.01	1280	0.22	0.05	0.46	0.04
0.02	0	0.11	0.02	40	0.09	0.04	0.22	0.07
0.03		1.04		160				
0	0.03	0.16	0.09	80	0.07	0.06	0.35	0.10
0.01	0	0.41	0	<20	0.38	0.01	0.39	0.11
0.02	0.02	0.36	0	20	0.19		0.53	0.03
0.01		0.62		640	0.09		0.17	
		1.08		1280	0.64		0.79	
0.04	0.04	0.42	0.12	80	0.16	0.12	0.20	0.04
0.07		0.74		<20				
0.27		0.35		40				
0.01	0.05	0.91	0.09	40	0.19	0.05	0.50	0.16
0.04		1.42		160				
0.09	0.03	0.20	0.07	20	0.17	0.08	0.42	0.14
0.08		0.39		160				

The ELISA technique is much more sensitive than agglutination. It also presents an easy way to analyze pertussis-specific antibodies in different immunoglobulin classes. Although this study used whole pertussis cells as an antigen, the same method could be used to measure a response to different cellular components.

The preimmunization level of antipertussis antibodies is very low according to both ELISA and agglutination, which could be a proof of the specificity of ELISA. Three injections of DTP vaccine increased the mean A_{400} value of antipertussis IgG 12-fold (Fig. 3), and the geometric mean titer of agglutination from <20 to 120. It is noteworthy that the only infant with poor antipertussis IgG response had the highest preimmunization value ($A_{400} = 0.27$), presumably due to antibodies of maternal origin. The significant reduction of anti-pertussis antibodies during the first year and the positive effect of booster injection are shown in Figure 3. This strengthens the view that if three DTP injections are given at the age of 2–6 months, a booster injection is necessary 1 year later.

The very low amount of antipertussis IgM after the basic immunizations could be explained by the late blood samples i.e. 2 months after vaccination, and the immunological immaturity of children at the age of 2–6 months.

REFERENCES

1. Lacey, B. W. Cultural diagnosis of whooping cough. Bull. WHO 23:31–35; 1960.
2. Cohen, S. M.; Wheeler, M. W. Preparation of pertussis vaccine in fluid medium. WHO Tech. Rep. Ser. 61:82–88; 1953.
3. Lautrop, H. Laboratory diagnosis of whooping-cough or *Bordetella* infections. Bull. WHO 23:15–31; 1960.

4. Preston, N. W. Potency test for pertussis vaccines: doubtful value of intracerebral challenge test in mice. J. Pathol. 91:173–179; 1966.

5. Chalvardjian, N. The content of antigens 1, 2, and 3 in strains of *Bordetella pertussis* and in vaccines. Can. Med. Assoc. J. 92:1114–1116; 1965.

6. Bronne-Shanbury, C. J.; Miller, D.; Standfast, A.F.B. The serotypes of *Bordetella pertussis* isolated in Great Britain between 1941 and 1968 and a comparison with the serotypes observed in other countries over this period. J. Hyg. (Camb.) 76:265–275; 1976.

7. Preston, N. W.; Mackay, R. I.; Bamford, F. N.; Crofts, J. E.; Burland, W. L. Pertussis agglutinins in vaccinated children: better response with adjuvant. J. Hyg. (Camb.) 73:119–125; 1974.

8. Dolby, J. M.; Stephens, S. Pertussis antibodies in the sera of children exposed to *Bordetella pertussis* by vaccination or infection. J. Hyg. (Camb.) 71:193–207; 1973.

9. Holt, L. B. The culture of *Bordetella pertussis*. J. Gen. Microbiol. 27:323–325; 1962.

10. Engvall, E.; Perlmann, P. Enzyme-linked immunosorbent assay, ELISA. J. Immunol. 109:129–135; 1972.

11. Preston, N. W. Effectiveness of pertussis vaccines. Br. Med. J. 2:11–13; 1965.

12. Preston, N. W. Prevalent serotypes of *Bordetella pertussis* in non-vaccinated communities. J. Hyg. (Camb.) 77:85–91; 1976.

13. Abbott, J. D.; Preston, N. W.; Mackay, R. I. Agglutinin response to pertussis vaccination in the child Br. Med. J. 1:86–88; 1971.

In vitro Model of *Bordetella pertussis* Infection: Pathogenic and Microbicidal Interactions

K. E. Muse, D. Findley, L. Allen, and A. M. Collier

ABSTRACT

Hamster tracheal organ culture was employed as a model for study of the pathogenesis of infection due to *Bordetella pertussis*. Exposure of organ cultures to Phase I *B. pertussis* (strain 114) results in a progressive cytopathology in the ciliated epithelial cell population, epithelial cell extrusion from the mucosal surface, and eventual necrosis. Ciliary activity, measured stroboscopically, was inhibited. Tracheal organ cultures maintained in filtered media from infected organ cultures behaved as uninfected cultures in terms of ciliary activity, suggesting bacterial attachment to target cells is required for pathogenesis. The ciliary activity index has been further used as an assay to determine the degree of virulence of multiple phase I strains.

Work done in collaboration with Dr. Ezzell and colleagues showed that SDS-polyacrylamide gel electrophoresis profiles of cell-envelope polypeptides of phase I and phase IV organisms were remarkably similar except for the absence of two high molecular weight and one low molecular weight polypeptide in the phase IV strain. Phase I organisms grown in a high magnesium medium (40 mM) produced antigenically modulated cells that gave a cell-envelope polypeptide profile indistinguishable from phase IV organisms. These organisms were equally effective in producing ciliostasis in tracheal organ cultures, as were the normal phase I organisms. However, this comparative difference provides an experimental parameter to further assay surface components as a virulence attribute that facilitates bacterial attachment to target cells.

In vitro experiments were performed to study the phagocytic and bactericidal capabilities of guinea pig alveolar macrophage (AM) for *B. pertussis*. Alveolar macrophage monolayers were challenged with phase I organisms in the presence of preimmune and anti-*B. pertussis* serum. Stained preparations were examined light microscopically and the phagocytic index calculated; a mean of 10% and 42% respectively of the AM contained bacteria. The phagocytic index for phase IV organisms in the presence of preimmune serum was calculated to be 48%. The phagocytosis of *B. pertussis* was significantly reduced at low temperatures (4° C) and by the microfilament-disruptive agent, cytochalasin B, suggesting the importance of cytoplasmic contractile proteins in the process. The viability of phagocytically active AM resembled that of unchallenged AM. The intracellular killing rate was similar for both phase I and phase IV organisms. The phagocytosis and degradation of *B. pertussis* by guinea pig AM was further assessed by scanning and transmission electron microscopy.

These data suggest that the interaction of *B. pertussis* and AM is a potentially important aspect of disease pathogenesis and is facilitated by certain immunologic factors.

INTRODUCTION

Bordetella pertussis is an important pathogen of the human respiratory tract, particularly in young infants (1). Because man is the only natural host of *B. pertussis*, experimental animal (2–5) and in vitro models (6–9) have been used to study the pathogenesis.

In the natural human (10) and animal model disease, *B. pertussis* demonstrates a striking tropism, colonizing the mucosal surface of the trachea and bronchi and producing a localized infection. In tracheal organ cultures, the pathogen exhibits a noninvasive surface infection with a pronounced tropism for the ciliated respiratory epithelial cells,

resulting in altered metabolism (8) and marked cytopathology (9).

Several studies have discussed the mechanisms by which pathogenic bacteria attach to mucosal surfaces and induce host cell injury at the site of infection (11–15). In vitro studies (9,10) with hamster tracheal organ cultures infected with virulent *B. pertussis* have demonstrated a correlation between the selected attachment to ciliated respiratory epithelium and abilities to produce symptoms of *B. pertussis* infection in vitro.

In the lung's defense against respirable particles that reach the distal airways, alveolar macrophages assume major importance. They are known to

actively ingest and degrade a variety of infectious organisms (16–18). Present evidence suggests that humoral antibodies, which act as opsonins, mediate the discriminatory recognition of certain infectious microorganisms by pulmonary phagocytes, thus promoting phagocytosis and microbicidal activities (19).

The purpose of this study is to use hamster tracheal organ cultures to delineate the relationship between host-*B. pertussis* interaction and microbial pathogenicity and to examine the in vitro antibody-mediated phagocytosis and degradation of *B. pertussis* by hamster alveolar macrophages.

MATERIALS AND METHODS

Organ Cultures

Hamster tracheal organ cultures were prepared as previously described (9) and incubated in Eagle's Minimal Medium (MEM) supplemented with 0.22 gm of sodium bicarbonate and 10 units of penicillin/ml of medium at 37° C for 24 hours in an atmosphere of 95% air and 5% CO_2.

Microorganisms

Virulent *B. pertussis* strain 114 was obtained from C. Manclark of the Bureau of Biologics, Bethesda, Maryland. Avirulent strain F–28 was obtained from the Michigan State Public Health Office. Cultures were established by streaking *B. pertussis* onto Bordet-Gengou agar with 0.5 unit of penicillin/ml of medium (Grand Island Biological Co.). Incubation was at 37° C in an atmosphere of 95% air and 5% CO_2.

Initiation of Infection

To initiate infectivity, a quantity of *B. pertussis* growth was resuspended to an optical density of 0.030 to 0.035 (approximately 10^7 organisms/ml of medium) at 540 mm in Stainer-Scholte Medium (SSM) modified to contain 1.525 gm/liter in Tris base. Selected organ culture rings were washed in MEM lacking penicillin and immersed in 0.5 ml of the bacterial suspension for 3 hours at 37° C. Infected tracheal rings were washed in SSM and transferred to a maintenance medium composed of equal parts of SSM and 2×MEM.

Ciliary Activity

All tracheal rings were examined for normal ciliary movement and evidence of cytopathology by the use of an inverted microscope at a magnification of 200× and 400×. The baseline cilia beating frequency of each ring was measured stroboscopically (General Radio Strobotac Type 1531–AB) following the initial 3-hour infection period. Beats per minute were counted at triangular points on the ring and the average of the three readings recorded. Ciliary activity was subsequently monitored every 12 hours. The results of strain 114 and strain F–28 were plotted as percent of control activity.

Electron Microscopy

At various intervals tracheal rings were removed from petri dishes containing the organ cultures, washed with warm (37° C) phosphate-buffered saline, and fixed in 2.5% glutaraldehyde in phosphate buffer at pH 7.4 for 1 hour. Specimens were rinsed in 0.1 M phosphate buffer (4° C) for 12 hours and dehydrated through graded ethanol solutions. After transfer through a gradient of freon 113 in ethanol, specimens were dried to critical point with freon 13 in a Bomar SPC–50/EX apparatus (Bomar Corp., Tacoma, Washington). Samples were attached to specimen studs with Dag cement and subsequently coated with carbon and gold in a Polaron sputter coater (Polaron Equipment Ltd., Watford, England). The specimens were viewed with an ETEC Autoscan scanning electron microscope (ETEC, Haywood, California) operating at 10–20kV. The images were recorded on Polaroid 55P/N film (Polaroid Corp., Cambridge, Massachusetts).

For transmission electron microscopy, tracheal rings were fixed in glutaraldehyde as described above, washed in phosphate buffer, and subsequently fixed in 1% osmic acid in 0.1 M phosphate buffer for 1 hour at 4° C. The tissue was dehydrated in graded ethanol solutions and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate, and examined in a Siemens 1A electron microscope.

Alveolar Macrophage Culture

Hamsters were anesthetized by intraperitoneal injection of sodium pentobarbital and exsanguinated by intracardiac puncture. The lungs were lavaged with 100 ml of 0.01 M phosphate-buffered saline at pH 7.2 in 10-ml aliquots. The cells were concentrated by centrifugation for 10 minutes at 200 rpm, washed with Hanks' balanced salt solution, and resuspended in Eagle minimal essential medium (Flow Laboratories) containing 100 U of aqueous penicillin G per ml and 20% heat-inactivated fetal calf serum (Flow Laboratories). 2×10^6

cells were placed on glass cover slips in Lux petri dishes and incubated overnight at 37° in air with 5% CO_2

Phagocytic and Bactericidal Activities

Bacteria were incubated in Eagle medium with 20% fetal calf serum and a 1:50 dilution of either *B. pertussis* (strain 114) antiserum or broth control serum. Glass adherent macrophages were overlaid with 10^6 organisms/ml medium. The macrophages were chemically fixed at intervals and counted directly by light microscopy for engulfed bacteria.

The bactericidal activity for *B. pertussis* was determined as previously described by Abrutyn et al. (20).

RESULTS

Interaction of *B. pertussis* with Hamster Tracheal Organ Cultures

Ciliary Activity. Ciliary activity in hamster tracheal organ cultures infected with virulent *B. pertussis* (strain 114) inhibited ciliary rates with a dose-response effect (Fig. 1). A reduction in activity was apparent 12 hours after the initiation of infection, with complete ciliostasis at 24 hours with an inoculum of 10^7 organisms/ml of medium. Inocula of 10^6 and 10^5 organisms/ml produced ciliostasis after 48 hours. To measure the possible role of a released pertussis toxin on host cell function, uninfected rings were immersed in media filtrates obtained from infected cultures for 6 days with no apparent effect on ciliary activity. Hamster tracheal organ cultures infected with avirulent *B. pertussis* (strain F–28) exhibited ciliary rates comparable to uninfected control cultures (Fig. 2). Additional phase I

strains were subsequently employed to evaluate the potential of ciliary activity of tracheal organ cultures as a bioassay for organism virulence. The variable ciliostatic endpoints produced by four selected strains of phase I *B. pertussis* suggests this model possesses the sensitivity to delineate differences in organism virulence (Fig. 3). Antigenic modulated phase I strains (grown in the presence of 40mM Mg^{++}) induced ciliostasis at similar times as the normal phase I organisms.

Figure 2. Effect of phase IV *Bordetella pertussis* (strain F–28) on the ciliary activity of hamster tracheal organ cultures.
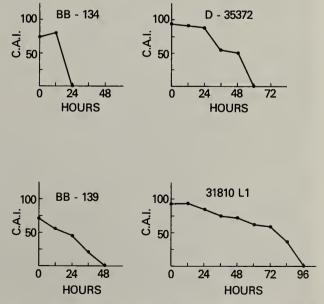
Figure 3. Effect of selected phase I *Bordetella pertussis* strains on ciliary activity of hamster tracheal organ cultures. Mean value from duplicate experiments infecting concentration was 10^7 organisms/ml.

Figure 1. Effect of phase I *Bordetella pertussis* (strain 114) on the ciliary activity of hamster tracheal organ cultures.

Ultrastructure of Host-Parasite Interactions. SEM of uninfected hamster tracheal rings maintained in culture for 24 hours showed the mucosal surface to be composed of two recognizable cell types, a predominant ciliated cell and a microvillous cell (Fig. 4A). Tracheal organ cultures infected for 24 hours with virulent *B. pertussis* (strain 114) showed a slight reduction in ciliary density relative to uninfected controls (Fig. 4B). Scanning electron micrographs showed that the initial contact between the
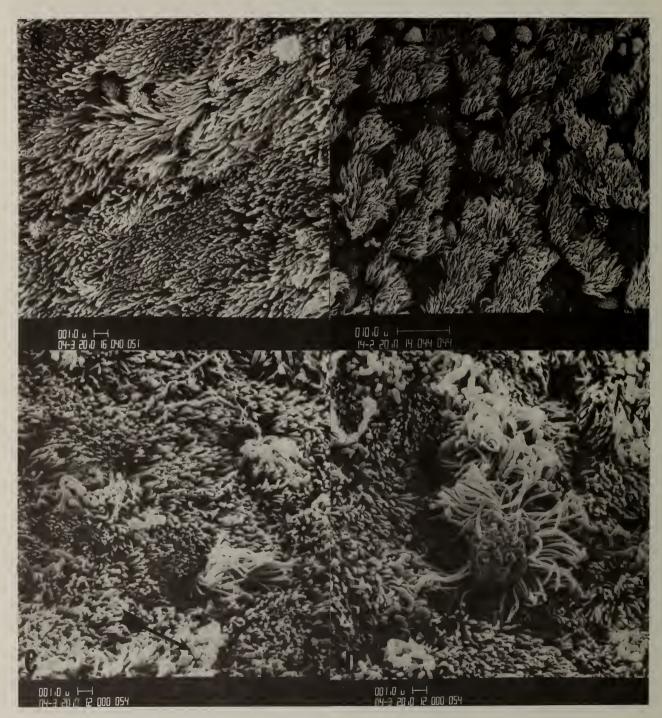
Figure 4. Scanning electron micrographs of (A) lumenal surface of uninfected hamster trachea after 24 hours in organ culture; (B) hamster tracheal organ culture infected with *B. pertussis* for 24 hours. Note reduced ciliated cell population relative to uninfected controls in (B). Hamster trachea infected for 24 hours showing (C) bacterial colonization (arrow) of mucosal surface; (D) partial exfoliation of infected ciliated epithelium.

infectious and mucosal surfaces was the ciliary apex which appeared at discrete cellular localities (Fig. 4C). Small numbers of ciliated cells were observed partially extruded from the lumenal surface as a result of the infection. These cells were characterized by disoriented cilia and adherent bacteria (Fig. 4D).

TEM of tracheal cultures infected for similar times showed that the organisms rapidly infiltrate the ciliary blanket to the base of the cilia. Electron micrographs show organisms associated with microvilli through filamentous glycocalyx components or in direct apposition with the microvillous or ciliary membrane (Fig. 5) producing firm attachment.
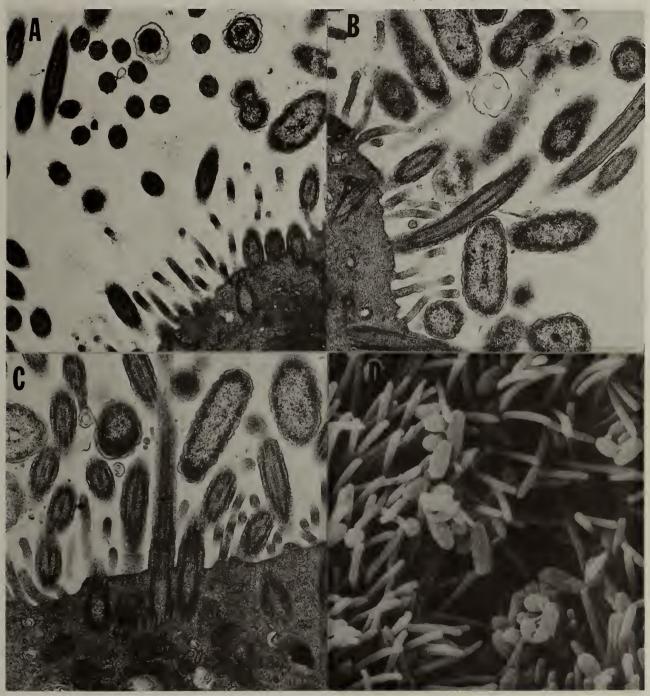
Figure 5. Transmission electron micrographs of (A) *B. pertussis*-infected trachea after 24 hours in organ culture. Note fibrillar glycocalyx components (arrow) bridging bacteria and microvilli. ×18,000. (B,C) Ciliated epithelial cell infected with *B. pertussis* for 24 hours. Note close apposition between bacteria and host cell membrane. ×20,000, × 22,000. (D) Scanning electron micrograph showing *B. pertussis* infiltration of ciliary layer.

These observations failed to confirm pili as a factor in the initial stages of *B. pertussis* attachment to host cells. Further TEM examinations of infected cultures showed that the virulent bacteria remain noninvasively adhered to the cell surface through-out the infectious cycle, even after epithelial cell exfoliation from the mucosal surface.

After a 48-hour infection period, the mucosal surface showed a marked reduction in ciliated cell density (Fig. 6A). This condition was paralleled by
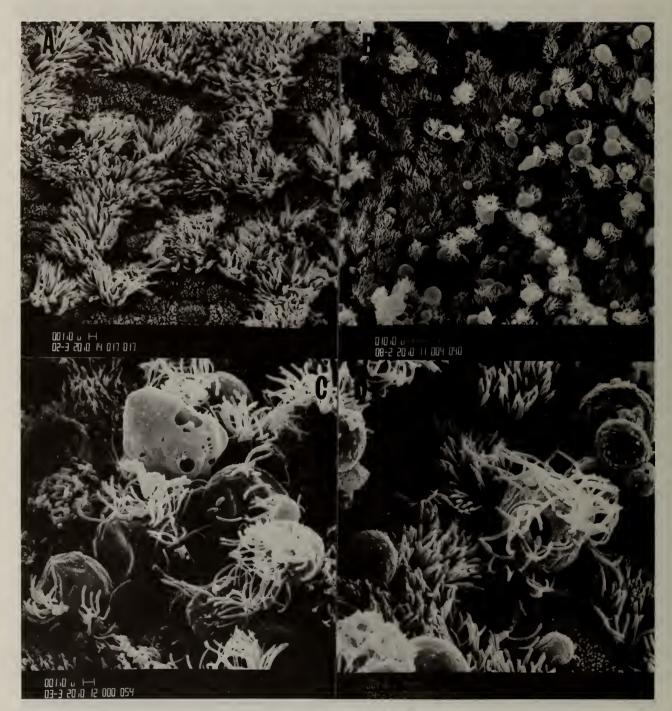
Figure 6. (A) Scanning electron micrograph of hamster trachea infected for 48 hours showing ciliated epithelial cell coloniza-tion by *B. p rtussis*. (B) Tracheal organ culture infected with *B. pertussis* for 48 hours. Note rounded, exfoliated cells over-lying mucosal surface. (C,D) Scanning electron micrograph showing altered, lysed state of exfoliated cells. Note bacteria at-tached to the cilia of these cells.

the exfoliation of infected cells and their subsequent accumulation on the epithelial surface (Fig. 6B–D). The remaining ciliated cells were heavily colonized by *B. pertussis* organisms while the microvillous cells remained free of adherent bacteria.

At 72 hours postinfection, the lumenal surface of the tracheal cultures consisted predominantly of microvillous cell types interspersed with occasional infected ciliated cells (Fig. 7A–C). Uninfected tracheal organ cultures and cultures infected with
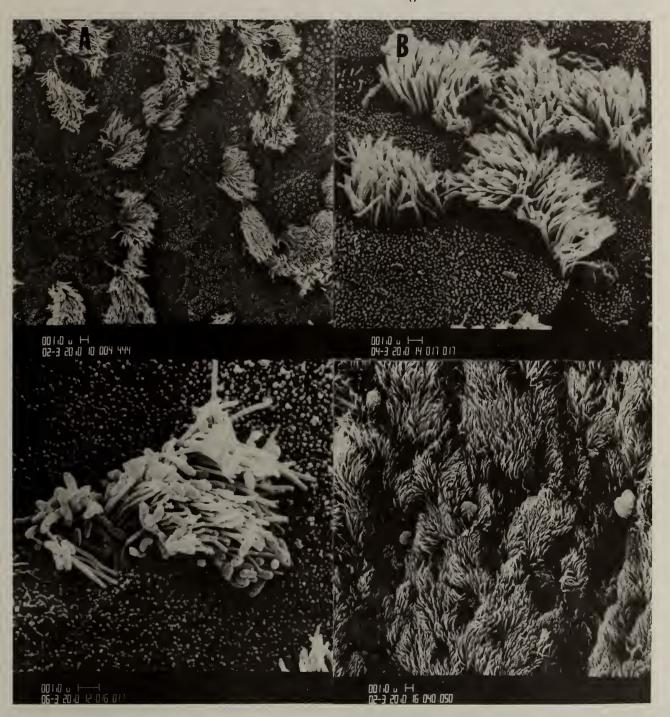
Figure 7. (A) Hamster trachea in organ culture infected with *B. pertussis* for 72 hours. Note large areas of nonciliated surfaces and their lack of bacterial parasitism. Scanning electron micrograph (B) showing colonization of ciliated epithelium 72 hours following infection; (C) of isolated ciliary tuft 72 hours postinfection. Note level of bacterial parasitism. (D) Scanning electron micrograph of hamster trachea infected with avirulent *B. pertussis* for 72 hours. Note normal morphology and absence of bacterial colonization.

phase IV *B. pertussis* were characterized by good preservation of normal cell surface morphology and the absence of surface-attached bacteria (Fig. 7D).

Interaction of *B. pertussis* with Alveolar Macrophages

Phagocytosis. Results of initial phagocytosis experiments indicated that guinea pig alveolar macrophages could phagocytize phase IV *B. pertussis* in vitro. On the basis of this observation, a study was undertaken to assay the biological conditions necessary to achieve maximum phagocytic and microbicidal activities in vitro.

B. pertussis, phases I and IV, were incubated with glass-attached alveolar macrophages at 37° C in the presence of nonimmune serum. At subsequent time intervals, the number of phagocytic cells engulfing bacteria was quantitized by direct counting using light microscopic techniques (Table 1). After 10 minutes, a mean of 25% of the macrophage population contained phase IV organisms while only 4% contained phase I *B. pertussis.* After 60 minutes, a mean of 48% contained phagocytized phase IV cells while less than 13% of the macrophages contained phase I organisms. Incubation of macrophages with phase I organisms in the presence of specific immune serum significantly enhanced phagocytic activity. By 60 minutes, 42% of the cells had been activated to engulf surrounding bacteria. At 4° C, the number of cells phagocytizing bacteria in each instance was less than 5%.

Bactericidal Activity. The ability of alveolar macrophages to kill viable *B. pertussis* was evaluated with the above model. Results, expressed as percent of phagocytized organisms killed, are presented in Figure 8. In the presence of nonimmune serum, 70% of the engulfed phase IV organisms and 5% of phase I organisms were killed. The bactericidal level for phase I organisms engulfed after opsonization was approximately 65%.

Figure 8. Effects of opsonization on the bactericidal capacity of alveolar macrophage for *B. pertussis* in vitro.

DISCUSSION

Organ culture has unique functional attributes that enhance its usefulness in pathogenicity studies. Most important is its maintenance of organized differentiated host cells under controlled conditions. Since the introduction of tracheal organ culture as an experimental system, it has been used extensively as a model for studying respiratory viral (21), mycoplasmal (22), and bacterial disease (23,24).

Ciliary activity is maintained in tracheal organ cultures and can be visually monitored. The ability to quantitate this activity stroboscopically permits monitoring injury and viability of epithelial cells. The exposure of tracheal organ cultures from chicks (6) and hamsters (8) to *B. pertussis* reportedly produced ciliostasis. In this study, the infection of hamster tracheal organ cultures with phase I *B. pertussis* inhibited ciliary activity with a dose-response effect. Accepting diminished ciliary activity as an index of *B. pertussis* virulence, we were able to categorize selected *B. pertussis* strains in descending order of virulence. Phase I cells antigenically modulated by growth in a high Mg^{++} (40 mM) medium exhibit altered envelope protein profiles (25) relative to control cells. The ciliostatic endpoint for cultures infected with such "C-mode" cells was comparable to control organisms. Studies are intended to further evaluate the possible role of these deleted proteins in pathogenicity. Ciliary activity in organ cultures infected with avirulent (phase IV) organisms remained identical to that of uninfected control cultures.

Attachment of *B. pertussis* to the host cell is a prerequisite in the pathogenesis of *B. pertussis* disease. These and previously reported results (8,9)

Table 1. Phagocytosis of *Bordetella pertussis*

Organism *Bordetella pertussis*	% Macrophage Phagocytizing B.p. Time (min)			
	0	10	30	60
Nonimmune serum (Strain *114*)	0 (0–75)	4 (3–75)	13 (10–75)	16 (12–75)
Antibacterial serum (Strain *114*)	2.8 (5–175)	21 (55–274)	28 (68–242)	42.3 (74–175)
Nonimmune serum (F-28)	4.7 (8–160)	25.7 (45–175)	35.1 (65–185)	48 (72–150)

show that virulent *B. pertussis* organisms attach selectively and noninvasively to ciliated respiratory epithelial cells in organ culture, resulting in altered cellular metabolism, reduction or cessation of ciliary activity, ciliated epithelial cell necrosis, and subsequent exfoliation from the mucosal surface. Hamster tracheal organ cultures infected with avirulent *B. pertussis* possessed no cell surface adherent bacteria and exhibited no detectable cytopathology. The fact that this pathological response occurs in organ cultures in the absence of an inflammatory response demonstrates the direct responsibility of the attached bacteria.

Bacteria are thought to attach to host cells mostly by London-van der Waals forces. For such forces to act, however, repulsive electrostatic charges on the host cell and bacterial surfaces must be overcome. A major hypothesis invoked to explain the attachment of bacteria to relevant mucosal surfaces is that it is mediated by 8-nm bacterial surface projections known as pili (11–15). Another explanation is that some capsular cell wall component also is an adhesive element. Such factors could account for known species or even host cell specificity.

A specific example is that of *Neisseria gonorrhoeae*, a Gram-negative organism that parasitizes mammalian mucosal surfaces. The organism possesses surface appendages (pili), which serve as a host cell attachment mechanism (26) as well as an antiphagocytic component (27). The loss of these surface appendages alters virulence.

B. pertussis has been reported to readily attach to various mammalian cell types in culture (27–29). The exact mechanism of *B. pertussis* attachment is questionable, however, although Morse and Morse (30) described surface filamentous projections similar to pili. Thus one can speculate that such elements are critical in the initial attachment of *B. pertussis* to host target cells. We have failed to observe such structural elements in the electron microscopic phases of this study. Further examinations therefore are required to confirm the mechanism(s) of *B. pertussis* attachment to host cells. The successful identification and isolation of such components responsible for attachment could be used in the future to develop highly specific vaccines to prevent host-parasitic interactions.

The unique predilection of virulent organisms for the cilia of specific tissues and the subsequent pathological abnormalities observed only in the individual parasitized cells lend support to the apparently diverse nature of determinants of virulence of *B. pertussis*. Further studies with the tracheal organ culture model should provide insights into the mechanisms by which organisms attach and mediate host cell injury at the site of infection. Such data may clarify the relationship between the localized parasitism and the observed disseminated host response to infection.

The mechanisms whereby acute *B. pertussis* disease is terminated are only partially understood. Evidence suggests that both humoral and cellular immunity factors are required for host recovery (1). *B. pertussis* is an extracellular pathogen vulnerable to pulmonary phagocytes. Indirect evidence from in vivo infectivity models (31,32) suggests that the interaction between virulent *B. pertussis* and alveolar macrophages may play an important role in disease pathogenesis and immunity. Under the in vitro conditions in these studies, hamster macrophages are activated by specific antiserum to phagocytize and kill virulent organisms. The avirulent organisms were readily engulfed and degraded in the absence of opsonization.

It has been reported that in *N. gonorrhoeae* the pili probably serve as an antiphagocytic component (27). There is no current evidence to document the antiphagocytic nature of such components in *B. pertussis*, and little is known about the significance of such a mechanism to disease pathogenesis.

ACKNOWLEDGMENTS

REFERENCES

1. Olson, L. C. Pertussis. Medicine 54:427–469; 1975.
2. Sprunt, D. H.; Martin, D. S.; McDearman, S. Results of the intratracheal injection of the Bordet-Gengou bacillus in the monkey and rabbit. J. Exp. Med. 67:309–322; 1938.
3. Mallory, F. B.; Hornor, A. A.; Henderson, F. F. The relation of the Bordet-Gengou bacillus to the lesion of pertussis. J. Med. Res. 27:391–397; 1913.
4. Burnet, F. M.; Timmins, C. Experimental infection with *Haemophilus pertussis* in the mouse by intranasal inoculation. Br. J. Exp. Pathol. 18:83–90; 1937.
5. Gallavan, M.; Goodpasture, E. N. Infection of chick embryos with *H. pertussis* reproducing pulmonary lesions of whooping cough. Amer. J. Pathol. 13:927–938; 1937.
6. Iida, T.; Ajiki, Y. Growth characteristics of *Bordetella pertussis* in chick tracheal organ culture. Jpn. J. Microbiol. 18:119–126; 1974.
7. Iida, T.; Ajiki, Y. The effect of 2,4-dinitrophenol on the growth of *Bordetella pertussis* in chick tracheal organ culture. Jpn. J. Microbiol. 19:381–386; 1975.
8. Baseman, J. B. Pathogenesis of *Bordetella pertussis* infection in hamster tracheal organ culture. J. Infect. Dis. 136:9196–9203; 1977.

9. Muse, K. E.; Collier, A. M.; Baseman, J. B. Scanning electron microscopic study of hamster tracheal organ cultures infected with *Bordetella pertussis*. J. Infect. Dis. 136:768–777; 1977.

10. Pittman, M. *Bordetella pertussis*—bacterial and host factors in the pathogenesis and prevention of whooping cough. Mudd, S., ed. Infectious agents and host reactions. Philadelphia: W. R. Saunders Co.; 1970: 239–270.

11. Word, M. E.; Watt, P. J.; Robertson, J. N. The human fallopian tubes: A laboratory model for gonococcal infection. J. Infect. Dis. 129:650–659; 1974.

12. Freter, R.; Jones, G. U. Adhesive properties of *Vibrio cholerae*: nature of this interaction with intact mucosal surfaces. Infect. Immun. 14:246–256; 1976.

13. Sranborg, E.; Hanson, C. L. A.; Jodal, U.; Lindberg, U.; Akerlund, A. S. Variable adherence to normal human urinary tract epithelial cells of *E coli* strains associated with various forms of urinary tract infection. Lancet ii:490–492; 1976.

14. Moon, H. W.; Nagy, B.; Isaacson, R. E. Intestinal colonization and adhesion by enterotoxigenic *Escherichia coli*: ultrastructural observations on adherence to ileal epithelium of the pig. J. Infect. Dis. 136:5124–5129; 1977.

15. Isaacson, R. E.; Fusco, P. C.; Brinton, C. C.; Moon, H. W. In vitro adhesion of *E coli* to porcine small intestinal cells: Pili as adhesive factors. J. Infect. Immun. 21:392–397; 1978.

16. Green, G. M.; Kass, E. H. The role of the alveolar macrophages in the clearance of bacteria from the lung. J. Exp. Med. 119:167–175; 1964.

17. Green, G. M. In defense of the lung. Am. Rev. Respir. Dis. 102:691–703; 1970.

18. Goldstein, E.; Lippert, W.; Worshauer, D. Pulmonary alveolar macrophage: Defender against bacterial infection of the lung. J. Clin. Invest. 54:519–528; 1974.

19. Green, G. M. Pulmonary clearance of infectious agents. Ann. Rev. Med. 19:315; 1968.

20. Abrutyn, E.; Solomons, N. W.; St. Clair, L.; MacGregor, R. R.; Root, R. K. Granulocyte function in patients with chronic renal failure: Surface adherence, phagocytosis, and bactericidal activity in vitro. J. Infect. Dis. 135:1–7; 1977.

21. Klein, J. D.; Collier, A. M. Pathogenesis of human parainfluenza type 3 virus infection in hamster tracheal organ culture. Infect. Immun. 10:883–888; 1974.

22. Collier, A. M.; Baseman, J. B. Organ culture techniques with mycoplasmas. Ann. N. Y. Acad. Sci. 225:277–289; 1973.

23. Denny. F. W. Effect of a toxin produced by *Haemophilus influenzae* on ciliated respiratory epithelium. J. Infect. Dis. 129:93–100; 1974.

24. Baseman, J. B.; Collier, A. M. Diphtheria pathogenesis in guinea pig tracheal organ culture. Infect. Immun. 10:1146–1151; 1974.

25. Parton, R.; Wardlow, A. C. Cell envelope proteins of *Bordetella pertussis*. J. Med. Microbiol. 8:47; 1975.

26. Swanson, J. Studies on gonococcus infection. IV. Pili, their role in attachment of gonococci to tissue culture cells. J. Exp. Med. 137:571; 1973.

27. Punsalung, A. P., Jr.; Sawyer, W. D. Role of pili in the virulence of *N. gonorrhaeae*. Infect. Immun. 8:255; 1973.

28. Crawford, J. G.; Fishel, C. W. Growth of *B. pertussis* in tissue culture. J. Bacteriol. 77:465; 1959.

29. Felton, H. M.; Gaggers, G.; Pomerat, C. M. Reactions of cells in tissue culture to *Hemophilus pertussis*. I. Effect of whole organisms on brain tissue and results of treatment with specific and normal serum. Tex. Rep. Biol. Med. 12:960; 1954.

30. Morse, J. H.; Morse, S. I. Studies on the ultrastructure of *Bordetella pertussis*. I. Morphology, origin, and biological activity of structures present in the extracellular fluid of liquid cultures of *Bordetella pertussis*. J. Exp. Med. 131:1342–1357; 1970.

31. Iida, T.; Kusano, N.; Yamamoto, A.; Konosu, M. An immunofluorescence study of the action of antibody in experimental intracerebral infection of mice with *Bordetella pertussis*. J. Pathol. Bacteriol. 92:359; 1966.

32. Voino-Yasenetsky, M. V.; Khai, L. M. Development of the infectious process in relation to conditions of inoculation. J. Hyg. Epidemiol. Microbiol. Immunol. 4:405; 1960.

Biological Significance of *Bordetella pertussis* Fimbriae or Hemagglutinin: A Possible Role of the Fimbriae or Hemagglutinin for Pathogenesis and Antibacterial Immunity

Y. Sato, K. Izumiya, M.-A. Oda, and H. Sato

ABSTRACT

Filamentous hemagglutinin (HA) obtained from the culture supernatant of *Bordetella pertussis* phase I bacteria elicited mouse protective antigen activity. Antipertussis-HA serum gave passive protective immunity to mice against intracerebral or aerosol infections. The location of pertussis HA on bacterial cells was studied by electron microscopy, and it was concluded that the filamentous HA is derived from fimbriae on the bacterial surface. Studies with the scanning electron microscope showed that virulent, hemagglutinable pertussis phase I bacteria adhere strongly to many kinds of mammalian cell membranes, but that avirulent, nonhemagglutinable phase III bacteria are not adhesive. Adhesion of the organisms to the cell membrane was prevented by antipertussis-HA serum.

These in vivo and in vitro studies suggest that pertussis fimbriae or hemagglutinin play an important role in the pathogenesis of *Bordetella pertussis* and that antihemagglutinin is one of the important protective antibodies.

Previously we reported the characterization of leukocytosis-promoting factor (LPF) purified from the culture supernatant of phase I *Bordetella pertussis* by consecutive steps of ammonium sulfate fractionation, electrophoresis, and sucrose density gradient centrifugation (1,2). This original LPF preparation elicited leukocytosis-promoting (LP), histamine-sensitizing (HS), hemagglutinating (HA), and mouse protective antigen (MPA) activities (3).

Recently we separated this original LPF preparation into two fractions by repeated agarose column chromatography (4). One fraction, hereafter referred to as LPF, possessed strong LP and HS activities. The molecular weight of LPF was about 105,000, estimated by polyacrylamide gel electrophoresis and sucrose density gradient centrifugation (4). The electron micrograph of LPF derived from the culture supernatant of strain Tohama exhibits spherical molecules about 6 nm in diameter (Fig. 1). The correlation between several different biological activities exhibiting LPF, such as adjuvant, insulin secretive, leuko- and hemagglutinating, in addition to LP and HS activities, will be reported elsewhere.

The other fraction, referred to hereafter as HA, had high hemagglutinating activity and consisted of protein. The molecular weight was approximately 130,000, estimated by polyacrylamide gel electrophoresis and ultracentrifugation (4). The electron micrograph of HA (Fig. 2) shows homogenous filamentous molecules about 2×40 nm in size.

Mouse protective antigen activity was re-examined with each of these two highly purified fractions derived from original LPF possessing high protectivity to mice (Table 1).

Immunizing materials were treated with formalin as described previously (3). After treatment, the antigen solution was mixed with $AlPO_4$ gel, except for NIH Standard V (Japanese standard for the potency determination of pertussis vaccine). Each of these precipitated antigens was diluted in phosphate-buffered saline to appropriate concentrations and was injected subcutaneously into 4-week-old SPF mice (strain DDY). After 21 days, each mouse was challenged with 18323. The mean effective dose (ED_{50}) was calculated by the method of Wilson and Worcester (5), and the protective potency was expressed in International Protective Units (IPU) per mg of protein of the immunized material. NIH Standard V was used as a reference for potency; the ampule contains 360 IPU/500 IOU (International Opacity Units). (1.0 IOU corresponds to 0.72 IPU as indicated in Table 1.) All HA preparations elicited mouse protective activities ranging from 32.1 to 150.5 IPU/mg of protein, with an average of about 90 IPU/mg of protein.
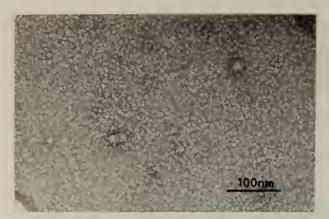
Figure 1. Electron micrograph of LPF molecules purified from the supernatant fluid of a liquid culture of *Bordetella pertussis* phase I, strain Tohama. A 1% solution of uranyl acetate in distilled water, adjusted to neutrality, was used for negative staining. Average diameter of the LPF spherical molecules is about 6 nm.

Figure 2. Electron micrograph of HA molecules purified from the supernatant fluid of a liquid culture of *Bordetella pertussis* phase I, strain Tohama. A 1% solution of uranyl acetate was used for staining. Homogenous filamentous molecules of about 2 × 40 nm are seen.

The results indicate that the protective antigen activity present in original LPF was carried with the filamentous HA, not with LPF or HSF itself. Table 2 shows the result of the mouse protection test with HA obtained from the culture supernatant of four different serotype strains: 353/Z, Tohama, No–134 and 18323. All of these HAs also elicited high protection in mice against intracerebral challenge with strain 18323. These results suggest that the filamentous HA antigen is one of the mouse protective antigens of *Bordetella pertussis*. Keogh and North (6) reported the protective antigen and hemagglutinin of *Bordetella pertussis* were the same substance.

Purified HA readily adheres not only to the red blood cells of many kinds of animals but also to many other mammalian cells manifesting agglutination. Figure 3 is an electron micrograph of a thin section of agglutinated sheep red blood cells incubated with pertussis filamentous HA purified from strain Tohama. Cell agglutination by the filamentous HA molecules is achieved by end-to-end linkage of two molecules of HA. There was no influence on the agglutinin titers of the HA with sheep red blood cells either before or after treatment of the cells with trypsin or neuraminidase.

A hemagglutination inhibition (HI) test was performed with several substances showing inhibitory activity for Gram-negative bacterial HA or phytohemagglutinin. It was observed that the most potent HI activity was elicited by cholesterol. Experiments with artificial membranes or liposomes prepared by mixing with cholesterol or closely related sterols and phospholipid suggested that one kind of receptor on the cell membrane for the filamentous HA of pertussis consists of sterols with a cis-hydroxyl at the 3-position. It also is possible that the side chains of the sterol influence the inhibitory activity of liposomes by determining the manner of insertion of the steroid nucleus into the lipid bilayer.

Hemagglutination of pertussis HA also was inhibited by normal, nonimmunized serum. This nonspecific HA inhibition was due to low density lipoproteins containing cholesterol in their lipid moeity. To obtain the specific antibody titer to pertussis HA it was necessary to remove the nonspecific inhibitor from test serum by treatment with kaolin or acetone before doing hemagglutination inhibition tests.

Next we investigated the location of the filamentous HA in pertussis bacteria. As is well known, the fimbriae of some Gram-negative bacteria have hemagglutinating activity. Phase I virulent pertussis strains with hemagglutinating activity were studied with the electron microscope. Figure 4, a micrograph of strain Tohama phase I cells, shows many filamentous fimbriae on the cell surface. Figure 5, a micrograph of strain 134 (so-called Pillemer's strain) phase I cells, shows the fimbriae more clearly. On the other hand, we have not observed fimbria-like structures with avirulent nonhemagglutinable phase III strains Tohama and 134. Morse and Morse also reported the presence of fimbria-like structures on the surface of *Bordetella pertussis* (7).

By using a Sepharose 4B column conjugated with purified pertussis HA, we prepared specific

Table 1. Mouse Protection Tests of Purified HA and LPF Obtained by Repeated Agarose Gel Filtration of Original-LPF

Expt	Immunizing Materials	Dose Inoculated (μg of protein per mouse)	S/16	ED$_{50}$(1 SD; μg of protein)	Potency (IPU/mg of protein)
	Original-LPF	15	13	5.2	52.0
		3	5	(3.9–7.0)	
		0.6	1		
	HA	15	12	8.4	32.1
		3	2	(6.6–10.6)	
I		0.6	0		
	LPF	15	4	—	—
		3	0		
		0.6	1		
	NIH Standard V	1.5 IOU	14	0.37 IOU	0.72 IPU/1.0 IOU
		0.3	6	(0.28–0.50)	
		0.06	2		
	Original-LPF	15	14	2.7	123.0
		3	8	(1.9–3.7)	
		0.6	3		
	HA	15	15	2.2	150.5
		3	9	(1.6–2.9)	
II		0.6	3		
	LPF	15	3	—	—
		3	1		
		0.6	0		
	NIH Standard V	1.5 IOU	14	0.46 IOU	0.72 IPU/1.0 IOU
		0.3	5	(0.35–0.60)	
		0.06	0		
	Original-LPF	15	14	2.4	102.0
		3	10	(1.8–3.2)	
		0.6	2		
	HA	15	15	2.4	102.0
		3	8	(1.8–3.2)	
III		0.6	3		
	LPF	15	3	—	—
		3	2		
		0.6	0		
	NIH Standard V	1.5 IOU	15	0.34 IOU	0.72 IPU/1.0 IOU
		0.3	4	(0.24–0.47)	
		0.06	4		

The immunizing materials were treated with formalin, mixed with AlPO$_4$ gel, then injected subcutaneously into mice. Three weeks after the antigen injection, mice were challenged with about 200 LD$_{50}$ of strain 18323.

pertussis HA antibody from the hyperimmunized anti-HA rabbit serum to see if the specific HA antibody could react with the fimbriae on pertussis phase I bacteria. Strain Tohama phase I bacteria were suspended in PBS at a concentration of about 10^9/ml, and 50μl of the suspension was added either to 50μl of normal rabbit γ-globulin solution (2mg protein/ml) or to 50 μl of the specific HA antibody solution (1.9 mg protein/ml). The mixtures, on sheets of "Parafilm," were left at room temperature for 10 minutes. Twenty μl of the mixture was then transferred to a 5-mm diameter disk of membrane filter, and unbound antibody or γ-globulin molecules were removed by suction. The organisms were then washed by distilled water, transferred to an electron microscopy specimen grid, and stained with a 1% solution of uranyl acetate.

Table 2. Mouse Protective Tests with Purified HA Obtained From Several Different Serotype Strains

Immunizing Material (purified from strain)	Serotype of the Strain	Dose Inoculated (μg of protein per mouse)	S/16	ED_{50}(1 SD; μg of protein)	Potency (IPU/mg of protein)
HA (Strain 353/Z)	1.	15	11	10.4	42.9
		3	1	(8.4–12.9)	
		0.6	0		
HA (Strain Tohama)	1.2	15	14	6.2	72.1
		3	2	(4.8–7.9)	
		0.6	1		
HA (Strain No–134)	1.3	15	11	7.6	58.8
		3	4	(5.4–10.7)	
		0.6	1		
HA (Strain 18323)	1.2.3	15	15	5.4	82.7
		3	2	(4.3–6.8)	
		0.6	1		
NIH Standard V		1.5 IOU	13	0.62 IOU	0.72 IPU/
		0.3	4	(0.48–0.73)	1.0 IOU
		0.06	0		

These HA solutions were treated with $AlPO_4$ gel except for NIH Standard V, then injected subcutaneously into mice. Three weeks after immunization, mice were challenged intracerebrally with about 200 LD_{50} of strain 18323.

Figure 6, an electron micrograph of Tohama phase I bacteria mixed with the specific Tohama-HA antibody solutions, shows that the specific HA antibody molecules uniformly attached to and reacted with the bacterial fimbriae. However, when normal rabbit γ-globulin solution was used instead of the specific HA antibody solution we observed no regular attachment or specific reations of γ-globulin molecules with the bacterial fimbriae. The results indicate that the filamentous HA molecules are derived from the fimbriae of the bacterial surface.
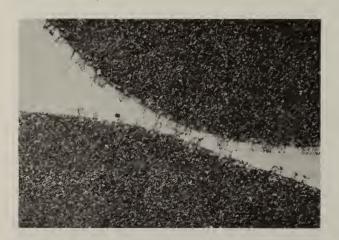
Figure 3. Electron micrograph of ultra-thin section of sheep red blood cells agglutinated by purified filamentous HA. Agglutination by filamentous HA molecules is attained by end-to-end attachment of two HA molecules.

Although the histopathology of whooping cough is known, little is documented about its bacterial pathogenesis. Chick (8) or hamster (9) tracheal organ culture has been employed as a model for the study of the pathogenesis of Bordetella pertussis infection. Bordetella pertussis initially grows on the mucous membranes of the respiratory tract, displaying a characteristic localization and colonization on the cilia of the epithelial cells. Recent studies in our laboratory show that pertussis phase I organisms that are virulent and hemagglutinable and possess fimbriae adhere readily and strongly to many kinds of tissue culture cells. Figure 7 is a photomicrograph of HeLa cells infected with phase I pertussis, strain Tohama. One-day-old HeLa monolayer cells were prepared in a small chamber containing 1 ml of fresh minimum essential medium (MEM). One-tenth ml of young B. pertussis cell suspension (10^{10}/ ml in MEM) was added to the monolayer cells, mixed gently, and allowed to settle for 2 hours in a CO_2 incubator. The monolayer cells were then washed throughly with MEM, fixed with ethanol, and Gram-stained with a 1% crystal violet solution. Stained preparations were layered with immersion oil and examined under a light microscope.

Much of the cell surface was covered with attached organisms. Figure 8 is a scanning electron micrograph of HeLa cells infected with phase I, strain Tohama. Infectious conditions were almost the same as those described above. Infected mono-

Figure 4. Electron micrograph of the fimbriae of *Bordetella pertussis* phase I, strain Tohama, 24 hr culture on B.G. medium stained with 1% uranyl acetate. Note numerous fimbriae on the cell surface.

Figure 6. Electron micrograph of the interaction of the specific pertussis HA antibody with bacterial fimbriae. *Bordetella pertussis* phase I, strain Tohama, 24 hr culture on B.G. medium, was mixed with specific HA antibody prepared by affinity chromatography from Tohama HA antirabbit serum. Uniform attachment of the specific HA antibody molecules to the bacterial fimbriae is seen.

layer cells on a glass cover slip were washed thoroughly with MEM, dehydrated through graded ethanol, and dried in a critical point drying apparatus. The cover slips were cut in half, mounted on metal stubs with silver paint, and gold coated. The specimens were then examined on a Hitachi Model S–430 scanning electron microscope (Hitachi, Ltd., Tokyo). Scanning electron microscopy dramatically reveals the attachment of *B. pertussis* phase I cells to HeLa cells (Fig. 8). However, phase III, strain Tohama, avirulent, nonhemagglutinable bacteria were not adhesive (Fig. 9).

We then examined the influence of HA antibody on the adherence of virulent phase I organisms to tissue culture cells. Five-hundredths ml of specific pertussis HA antibody solutions, prepared as above (1 mg of protein/ml; agglutinin titer, 2,560), and 0.1 ml of phase I cell suspensions (10^{10}/ml in MEM) were added to 1.0 ml of the HeLa cell cultures, mixed gently, then incubated for 2 hours. Specimens for light or scanning electron microscopy were prepared as stated above. Figure 10 is a photomicrograph of the HeLa cells treated with virulent pertussis cells and specific pertussis HA antibody solution. Adherence was loose, since there was a very small number of bacteria on the culture cells.

Figure 5. Electron micrograph of the fimbriae of *Bordetella pertussis* phase I, strain No–134 (Pillemer's strain), 24 hr culture on B.G. medium. The fimbriae are more clearly seen than in Figure 4.

Figure 7. Photomicrograph of HeLa cells incubated with *Bordetella pertussis* phase I, strain Tohama. After 2 hr incubation, the HeLa monolayer cells were washed thoroughly with MEM to remove free or nonadherent pertussis cells, then fixed and stained for light microscopic observation. Many pertussis bacteria are seen on the cytoplasmic membranes.

Figure 8. Scanning electron micrograph of HeLa cells infected with *Bordetella pertussis* phase I, strain Tohama. Much of the cell surface is covered with attached pertussis bacteria.

This indicates that pertussis HA antibody apparently can prevent the adhesive and colonizing activities of virulent phase I pertussis organisms. Anti-phase I whole-cell serum also prevented adhesion of phase I bacteria to tissue culture cells, but anti-
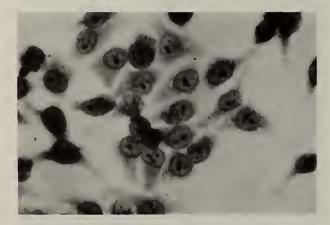
Figure 9. Photomicrograph of HeLa cells incubated with *Bordetella pertussis* phase III, strain Tohama. The infectious condition was the same as in the case of phase I, in Figure 7. No pertussis bacteria can be seen on the cytoplasmic membrane of the HeLa cells.
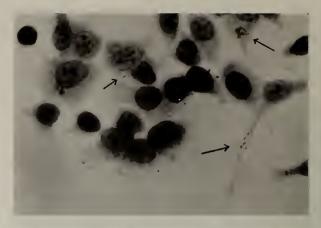
Figure 10. Photomicrograph of the HeLa cells infected with *Bordetella pertussis* phase I, strain Tohama. Specific anti-pertussis-HA antibody was added to the culture medium. Very few bacteria are seen on the infected HeLa cells.

phase III whole cell serum or anti-LPF(HSF) serum could not prevent the attachment.

In vivo, anti-HA rabbit serum gave passive protective immunity to mice against intracerebral challenge with virulent pertussis bacteria.

Recently, we developed inhalation equipment for respiratory infection of laboratory animals and found it suitable for study of pertussis infection in suckling mice younger than 10 days old. Phase I pertussis bacteria, suspended at a concentration of 10^{10}/ml in 1% casamino acid, were given to suckling mice by aerosol inhalation for about 60 minutes. True infection was demonstrated by recovery of up to 10^4-fold more viable pertussis cells from the lungs of aerosol-infected mice than from the lungs just after aerosol inhalation. Numbers of bacteria recoverable from the lungs of suckling mice at 30 minutes and at 7 to 10 days after the aerosol inhalation were approximately 10^5 and 10^9 per lung respectively. Typical signs of human whooping cough, such as severe leukocytosis, appeared in all inhalation mice a week after aerosol infection, and death occurred 2 weeks after inhalation. Figure 11 is a scanning electron micrograph of ciliated epithelial cells of a germ-free ICR mouse (10 days old at inhalation time) taken 7 days after aerosol inhalation of phase I pertussis, strain 18323. Many pertussis organisms are observed between the cilia of the epithelial cells. High resolution studies of ultra-thin sections (Fig. 12) suggest that virulent pertussis organisms are anchored by their fimbriae to the host cell surface. The inhalation system can be used to demonstrate passive protection test by HA-antibody; there were no detectable pertussis

Figure 11. Scanning electron micrograph of ciliated respiratory epithelial cells taken from a germ-free mouse 7 days after aerosol inhalation of *Bordetella pertussis* phase I, strain 18323. Many short rod-shaped pertussis cells are seen between the cilia of the epithelial cells.

Figure 12. Transmission electron micrograph of an ultra-thin section of *Bordetella pertussis*-infected ciliated respiratory epithelial cells. The experimental conditions were the same as in Figure 11.

cells 7 days after aerosol infection in mice that had received anti-HA serum before aerosol inhalation.

These in vivo and in vitro studies suggest that the pertussis fimbriae or hemagglutinin play an important role in the pathogenesis of *Bordetella pertussis,* and that hemagglutinin antibody is one of the important protective antibodies.

REFERENCES

1. Sato, Y.; Arai, H. Leukocytosis-promoting factor of *Bordetella pertussis*. 1. Purification and characterization. Infect. Immun. 6:899–904; 1972.

2. Sato, Y.; Arai, H.; Suzuki, K. Leukocytosis-promoting factor of *Bordetella pertussis*. II. Biological properties, Infect. Immun. 7:992–999; 1973.

3. Sato, Y.; Arai, H.; Suzuki, K. Leukocytosis-promoting factor of *Bordetella pertussis*. III. Its identity with protective antigen. Infect. Immun. 9:801–810; 1974.

4. Arai, H.; Sato, Y. Separation and characterization of two distinct hemagglutinins contained in purified leukocytosis-promoting factor from *Bordetella pertussis*. Biochim. Biophys. Acta 444:765–782; 1976.

5. Worcester, J.; Wilson, E. B. A table determining LD_{50} or the fifty per cent endpoint. Proc. Nat. Acad. Sci. U.S.A. 29:207–212; 1943.

6. Keogh, E. V.; North, E. A. The haemagglutinin of *Haemophilus pertussis*. I. Haemagglutinin as a protective antigen in experimental murine pertussis. Aust. J. Exp. Biol. Med. Sci. 26:315–322; 1948.

7. Morse, J. H.; Morse, S. I. Studies on the ultrastructure of *Bordetella pertussis*. 1. Morphology, origin, and biological activity of structures present in the extracellular fluid of liquid cultures of *Bordetella pertussis*. J. Exp. Med. 131: 1342–1357; 1970.

8. Iida, T.; Ajiki, Y. Growth characteristics of *Bordetella pertussis* in chick tracheal organ culture. Jpn. J. Microbiol. 18:119–126; 1974.

9. Muse, K. E.; Collier, A. M.; Baseman, J. B. Scanning electron microscopic study of hamster tracheal organ cultures infected with *Bordetella pertussis*. J. Infect. Dis. 136:768–777; 1977.

DISCUSSION OF PART 1

DR. CHRISTENSEN: Dr. Linnemann, you raised the question of the frequency of reinfection. It is an old experience that about 25% of mothers of infants with pertussis contract pertussis themselves. I have seen a few of these cases, quite severe.

Are there any comments on the paper read by Dr. Broome?

DR. DUDGEON: It has been suggested that some of the cases of pertussis, or paroxysmal coughs, in the epidemic in England are not due to *Bordetella pertussis*. I thought it might be of interest to give you some figures from my own hospital.

From July to October, 1978, of 21 admitted cases, *Bordetella pertussis* was isolated in 14. That is an isolation rate of 66% by culture. These were all severe cases; none of the patients had been vaccinated against pertussis. In our other hospital, Queen Elizabeth Hospital, of 110 cases, 78% were positive on culture. We were not able to isolate any viral agents, including adenovirus and parainfluenza virus.

A technical point is that all specimens were taken by two technicians, who processed all the swabs. In a study in the last 3 months, in 15 children all under 1 year, 13 were culture-positive. So in our experience, the overall isolation rate is of the order of 75% and I think a lot of it is due to good technique.

DR. J. B. ROBBINS: I have a comment and a suggestion for Dr. Broome's paper. To facilitate comparison of information regarding isolation techniques, would it not be worthwhile to specify the method for assay of potency, the fluorescein/protein ratio and the amount of protein necessary for direct staining of the slides so that the specificity, the potency, and the final concentration of the fluorescent conjugate can be compared between laboratories?

Would you also comment on whether there is any evidence that the individual animal used as a source of blood for the media has any influence on the ability to isolate the organism? Is this a factor that should be considered?

DR. BROOME: With regard to the characteristics of the conjugates used, the conjugate was identical when compared by the Oregon and Atlanta Laboratories, and was used in a comparable dilution. The specificity had been previously tested against positive controls for *Bordetella pertussis* and *Bordetella parapertussis*. The FP ratio was 28.6 μg of bound FITC per mg of protein per ml of conjugate, and the protein was 7.2 mg/ml.

There does seem to be an effect of the animal species of blood used for supplement in culture. We have used sheep's blood because of some studies that suggest larger colonies result from its use. The use of lysed defibrinated horse blood may result in a larger number of colonies, although possibly not as sizable ones. I think that reso-lution of which species to use would be helpful, particularly when you are talking about inocula from clinical samples that may have a very small number of organisms and further testing is necessary to clarify that issue.

DR. HEWLETT: I have several questions for Dr. Broome. Since the fluorescent antibody is used in the final confirmation of the cultural diagnosis in a number of cases, where is the weak link in the cases which are culture-positive and direct smear fluorescent antibody negative?

DR. BROOME: Well, I think there are several possible interpretations of the FA negativity in those samples. One would be that the smear preparation by a wide variety of personnel could have resulted in inappropriate thickness of smears and difficulty with reading and, in fact, the laboratory that was performing the test felt that a number of the smears were not optimal.

There is some question as to whether there may be increased sensitivity in picking up a smaller number of organisms by culture rather than by FA, because of the difficulty in examining a field as extensively as possible. All the smears were examined for 10 minutes before being declared negative, but even that may not result in picking up a very small number of organisms.

I think that those are some of the possible factors. We did do some biochemical confirmations as well; it was not a complete relying on immunofluorescence for confirmation of cultures.

DR. HEWLETT: Have there been any studies of the reliability of the zone of hemolysis around the typical colonies as a diagnostic criterion?

DR. BROOME: I think that is one of the concerns with variable sources of species for the blood in the media. We relied on colonial morphology, and not just on hemolytic appearance in selecting colonies to be processed for identification.

DR. KATZ: Dr. Broome, in the last paragraph of your presentation you mentioned that the Center for Disease Control was going to try to accumulate more and better data regarding the disease by the new surveillance form, which you did not show us. We also have seen recently a new form to try to assess reactions to DTP vaccine. I wonder if you have any data to suggest that increased numbers and length of forms produce better reporting, or if the Center might have some more imaginative ways to try to accumulate the data that hamper us all in trying to arrive at decisions.

DR. BROOME: I think that what we need are not more and longer forms, but better-designed forms that are directed toward answering specific questions. Some of those questions can best be answered on the basis of a national surveillance program that involves gathering widespread information from as many sources as possible, and some can be better answered by selected studies in areas where you have a high incidence of disease or good laboratory backup or whatever other circumstances may be important.

From the point of view of pertussis, I think we would like to get some estimate of the significance of the dis-

ease on a national scale in terms of morbidity and mortality. This has been something that has been rather inadequately answered on the basis of studies centered around a single outbreak or a single hospital. I think that the format of national surveillance can provide that sort of information.

For the longer, more complicated form addressed to specific outbreak situations, this would presumably be employed in a setting where an individual would wish to investigate an epidemic in detail and could use the form so that standardized information could be collected and analyzed from a number of different outbreaks.

DR. CHRISTENSEN: Are there any comments on the paper by Dr. Kuronen?

DR. HELTING: I have a question relating to the enzyme-linked immunosorbent assay which you were using. You coated your polystyrene tubes with whole bacterial cells. Have you tried to coat your cells with fractions extracted from bacterial cells to see whether antibodies are formed against specific fractions?

The second question is: You diluted your sera 1:500 in order to be able to measure the immune response in children receiving three or more injections. Is it possible to use the ELISA technique to evaluate the immune response after the primary immunization, after one injection, by diluting 1:10 or 1:20?

DR. KURONEN: The answer to the first question is that we haven't examined different fractions of pertussis, but that could be easily done, I suppose, because polystyrene is adsorbing most of the proteins, and that may be the future method.

In answer to the second question, it is possible to analyze samples after one injection by using lower dilutions.

DR. HELTING: May I add just one comment? We have looked at sera by ELISA from children immunized with pertussis using fractions or extracts from the bacteria, and we are getting immune response against certain fractions. The high molecular weight fractions are the most active ones.

DR. BLASKETT: Since about 1964 we have been monitoring the pertussis serotype makeup of infecting strains in Australia. We found that these followed the same pattern as in the rest of the world (Tables 1 and 2). Our old strains were type 1.2 predominantly, then we changed over to type 1.3. Since 1971, we have had a gradual

Table 1. Serotype Drift with Time

Years	Isolates	Serotypes		
		1.2 (%)	1.2.3 (%)	1.3 (%)
1950–69	41	73	20	7
1960–71	258	3	24	73
1971–75	83	30	26	44
1975–76	84	54	19	27
1976–77	45	49	18	33
1977–78	59	78	2	20
1978–79	33	91	3	6
Total	603			

Table 2. Distribution of Serotype 1.2 in Four Australian States

Years	Victoria	Western Australia	South Australia	Queensland	Total
1969–72	6/49	0/7	0/47	—	6/103
1972–75	12/21	6/10	3/30		21/61
1975–78	85/116	9/17	19/51	0/4	113/188
1978–79	23/24	3/4	2/3	2/2	30/33

resurgence of serotype 1.2, which is now our predominant infecting strain. I am encouraged by the fact that Dr. Kuronen has also found that type 1.2 is the predominant infecting strain in Finland.

DR. CHRISTENSEN: Are there any comments for Dr. Muse?

DR. COWELL: I was wondering whether growing the cells or treating the cells, either with mucin or some other glycoprotein, would have an effect on the antibody-mediated phagocytosis.

I have one other question. Since the transmission electron micrographs showed that there was very close association between the bacterial cells and the ciliated epithelial cells, I was wondering if there was any evidence of fusion between the membranes, and possibly transfer of material from the bacterial cell to the ciliated epithelial cells?

DR. MUSE: In answer to your second question, we have not looked at this, but we plan to do that, so I cannot answer yes or no. In regard to your first question, we have not done that.

DR. ORTEZ: In the tracheal system where you have ciliated epithelial cell destruction, is that destruction restricted, or are other cell types within the preparation also destroyed?

Secondly, in view of Dr. Sato's paper, have you put anything on this preparation that might, like sterols, have inhibited the binding of the bacterium to the cilia?

DR. MUSE: In regard to your first question, the primary effect seems to be, at least in terms of pathogenic effects, the ciliated epithelial cell. If we examine these cultures both by light microscopic and transmission EM techniques, the relationship does not appear to affect the microvillous cell types or subepithelial cell types.

The answer to your second question is that we have not done the experiments you suggested. We are interested in certain cell envelope proteins and their relationship in terms of attachment to host cells, but I think our work is very preliminary at this moment, and I would rather not comment on that.

DR. KLEIN: Regarding bactericidal capacity, it was not quite clear how that was determined. Was it done by means of lysing the cells after phagocytosis and looking for viable bacteria by dilution plating?

DR. MUSE: Yes.

DR. KLEIN: My second question has to do with what components of the immune serum you think are involved. Is it IgG? Do you think complement is involved? Is it IgM?

DR. MUSE: I would simply have to speculate on that. That is something that we have not analyzed. In the experiments that we have performed up to this date we have utilized an antiserum that we obtained from Dr. Manclark. We have not attempted to analyze the specific components involved.

DR. KLEIN: Is it hyperimmune serum, and has it been heat inactivated?

DR. MUSE: This is whole serum.

DR. KLEIN: My final comment is: Have you had an opportunity to look at the bactericidal capacity of alveolar cells from infected animals and their killing ability in the absence of immune serum components?

DR. MUSE: We have not.

DR. GOTSCHLICH: I have a question to ask you and afterward I would like to generate some discussion on your part. I gather that your impression is that the organisms are specific for the cilia of a hamster, and by inference, for the tracheal cilia of human beings. The question is: have you ever tried cilia from any other site? I would suggest to you the Fallopian tube, to see whether it is cilia-specific, or whether it is specific for the respiratory tract.

Second, would you comment on some of the results of Dr. Sato, particularly the adherence of bacteria to the HeLa cells, which I should not think are ciliated, and possibly the provocative suggestion of a cholesterol binding site.

DR. MUSE: All of the experiments that we have performed up to this date have been with tracheal organ cultures from the hamster. So we have not looked at any other sources of ciliated cells, even though these are experiments that we would like to do.

DR. J. B. ROBBINS: Just a further comment on Dr. Gotschlich's thought that other ciliated cells might be used. You know, the ependymal cells of the brain contain ciliated cells, but in humans it may be that a more convenient source of ciliated cells for studying adherence as well as effect on motion, which seems to be an important component of the pertussis adherence, might be spermatozoa. The flagella of spermatozoa functionally and anatomically seem to be similar to if not identical to respiratory ciliated cells. I suggest that an interesting reading might come from the work of Afzelius from Sweden, who has pointed out the curious association between immobile spermatozoa and the respiratory defect of Kartagener's syndrome, in which a functional and an anatomic lesion of cilia predispose to respiratory infections. It might prove an interesting and convenient source to study this attachment which, in my opinion, seems to be a critical pathogenic role for pertussis.

DR. MUNOZ: I was wondering if Dr. Muse has tested organisms such as Bordetella bronchiseptica in similar experiments. I am suggesting this because a number of years ago Dr. H. Felton found that in tissue cultures the heat-labile toxin was extremely toxic to these cells. I wonder if this toxin plays a role in the sloughing of cilia that you have demonstrated so beautifully.

DR. MUSE: We made a statement, I think, in our discussion, that we have looked at filtrates, filtered media from infected rings, and at least in terms of ciliostatic effects or observable cytopathology, we do not see that.

In terms of your other question, we have looked at the same organ culture model infected with B bronchiseptica and B. parapertussis, and with both of these organisms we see similar associations, attachment to ciliated cells, and cell sloughing. In the case of B. bronchiseptica, that we have looked at preliminarily, possibly some type of extracellular toxin may play a role, because we can demonstrate cytopathology and ciliostasis from filtered media from infected ring for B. bronchiseptica.

DR. PITTMAN: I would like to add a historical note which has not been published. When Dr. Pillimer first started preparation of his antigen, he adsorbed it onto cholesterol, and then he changed over to the red cell.

DR. COLLIER: I would like to comment on the attachment of Bordetella pertussis to differentiated cells. As far as attachment to ciliated ependymal cells of mouse brain, the one reported study in the literature states that B. pertussis organisms attached to ependymal cell microvilli and not to the cilia. In preliminary work we have examined the interaction of B. pertussis and human ciliated epithelium in organ culture. The epithelium was obtained by bronchial brush biopsies from human adults undergoing routine bronchoscopy. The tissue was maintained in liquid media and infected with virulent B. pertussis in vitro. The organisms attached to the ciliated cells with resulting injury similar to that we had observed and reported earlier in hamster tracheal organ cultures infected with B. pertussis (J. Infect. Dis. 136: S196–S203, 1977).

Dr. Sato, have you examined the effects of B. pertussis purified HA on ciliated cells in organ culture?

DR. SATO: I have no experience with that.

DR. WARDLAW: Dr. Muse, in view of the fact that very little work has been done with the hamster, I was wondering if there was some special reason for using it in preference to the mouse, where there is a great deal of information on respiratory tract infections.

DR. MUSE: No, the main reason is our past success with the hamster model.

DR. NOVOTNY: Dr. Muse, during your experiments with hamster cells were you of the opinion that the activity to attach to the cilia was from the side of bacteria or from the side of cilia, or can't you say?

DR. MUSE: We are attempting to design and perform some experiments that might answer that.

DR. COWELL: I was wondering if Dr. Sato tried to incubate or mix the phase I Tohama with the suspension of cholesterol, and then look at the binding to the HeLa cells. Does it prevent attachment?

DR. SATO: Cholesterol did not prevent attachment to the HeLa cells, because cholesterol is very difficult to keep in solution. So we used liposomes instead of cholesterol. But liposomes did not prevent attachment of the bacteria to the HeLa cells.

DR. GOTSCHLICH: Dr. Sato, as I understood your talk, the hemagglutinin is a single molecule, of which you showed a picture, of 130,000 molecular weight. Is that correct?

DR. SATO: Yes.

DR. GOTSCHLICH: In addition to that, you showed us pictures of fimbriae which exhibited on their surface the antigenic specificity of the hemagglutinin. How do

you account for this? Does the 130,000 molecular weight unit act as a subunit to assemble into a fimbria?

DR. SATO: I do not know.

DR. KONG: I have two questions for Dr. Sato. Is there any cross-reaction between your purified LPF and HA? How stable is your HA preparation?

DR. SATO: There is no cross-reaction between LPF and HA. As to the second question, it is not so stable.

DR. SCHUH: In your HA preparation, Dr. Sato, you have found the protective activity. In a publication of yours about 10 years ago you showed particles derived from the cell wall, and these particles had a sedimentation constant of 22S. You also found a protective activity in these particles?

DR. SATO: Yes.

DR. SCHUH: Is the protective activity somehow related in these two structures—is it the same antigen, or are these antigens different?

DR. SATO: The antigens are different, I think.

DR. MUNOZ: The paper of Dr. Sato is extremely important and interesting to me, but there are some things that we have to resolve between the two laboratories. We have prepared fully protective materials from cultures that have hardly any hemagglutinin, and just recently Dr. Arai, who presently is working in our laboratory, has shown that in shake cultures, the hemagglutinin is destroyed. These are the cells that we have always used to prepare pertussigen, which does protect.

In very recent experiments, Dr. Arai has confirmed that the hemagglutinin does protect mice.

So the possibility exists that there is more than one mouse-protective antigen in pertussis, and I think this problem should be very meticulously studied so that it can be solved.

By the way, the name pertussigen is given to a substance that does many things, but it is not the heat-labile toxin, and is not the heat-stable toxin. One of the slides projected in the first paper could have been misinterpreted as saying that pertussigen was everything that was in that slide, which is not correct.

DR. FOX: I did want to comment on some of the epidemiologic part of Dr. Linnemann's paper. This concerns the obvious shift in importance of infections among older people in the spread of the disease. There was a table that showed the apparent source of infection in a fair number of cases. In a certain proportion of these, it was younger children, and in another proportion it was adults. In a third proportion it was unknown. I think this is not unexpected when one considers that this is a respiratorially transmitted disease, and there is probably a good deal of cross infection going on in the community. If this was a household type study, this latter proportion probably represents individuals who brought it in on their own and did not spread it to others.

Part 2. *Bordetella pertussis*–GENETICS, PHYSIOLOGY, NUTRITION, GROWTH, VACCINE PRODUCTION

Chairman: *Charlotte D. Parker*
Rapporteur: *Dennis Stainer*

The Genetics and Physiology of *Bordetella pertussis*

C. D. Parker

Bordetella pertussis is a fascinating organism to study. A certain amount of empiricism, as opposed to logic, is required for success with pertussis. Diagnostic cultures are difficult and sometimes unreliable. Different lots of vaccine, made in the same way, from the same strains, sometimes show different properties. Experimental work is not always reproducible from one laboratory to another, but this is common in biological research. The diagnostic culture problems and the unexpected variability in vaccines and in pertussis strains themselves are not easy to explain.

Successful pertussis workers learn to perform diagnostic cultures and to conform to other empirical rules. They use huge inocula when transferring a pertussis strain. They keep stock cultures frozen or lyophilized to avoid passage. They return to stock cultures for each new experiment. They measure cell numbers by "opacity units" rather than by viable counts. I have asked myself why *B. pertussis* is so different from most Gram-negative eubacteria.

Is B. pertussis *different because it is nutritionally demanding?* The answer to this question is clearly negative. Both my own experience (1) and the literature make it clear that *B. pertussis* has only a few simple growth requirements. *Is* B. pertussis *different because it is so fastidious?* This question is helpful in explaining the difficulties found when growing *B. pertussis,* since we know many substances are inhibitory to *B. pertussis.* But this cannot be the entire answer, since other fastidious organisms such as *Brucella abortus, Neisseria gonorrheae,* etc., do not present the special problems that are routine with *B. pertussis. Is* B. pertussis *different because of some peculiarity in its genetics? Is* B. pertussis *different because of some peculiarity in its metabolism?* These are the two questions I address in this paper.

THE GENETICS OF *Bordetella Pertussis*

Several workers have examined the variability of *B. pertussis,* since this is one of the unusual properties of the organism. Leslie and Gardner (2) defined a series of antigenic changes in pertussis cultures, using the term phase I to indicate strains that resemble fresh isolates. Phases II, III, and IV were the result of progressive antigenic changes. Standfast (3) serially cultured fresh isolates of *B. pertussis* on Bordet-Gengou medium containing peptone and found that some strains acquired the ability to grow on 10% sheep blood agar. None acquired the ablity to grow on nutrient agar. He also investigated the biological and serological properties of these strains and of their serially passaged derivatives and found variation in the passaged strains, but no variation among the fresh isolates. However, the changes he noted were not systematic, but appeared to occur randomly. Changes in immunogenicity, virulence, toxic properties, hemagglutination, and growth properties appeared to vary independently.

These reports suggest that a series of loss mutations may occur. The report of Leslie and Gardner suggests that the change in antigens is systematic and invariable. Standfast concluded that antigenic changes, as well as other changes, occur in any order, but overall, the strains tend to lose the characteristics of fresh isolates. I have previously discussed evidence to support the hypothesis that degraded (or phase IV) strains represent mutants that have undergone a series of loss mutations in any order (1). The accumulation of such mutants in the population with relatively few transfers argues that selection in artificial culture is extremely severe. The extreme sensitivity of *B. pertussis* to fatty acids, peroxides, etc., as reviewed by Rowatt (4), makes such rapid in vitro change plausible.

One reasonable explanation for the variability in *B. pertussis* can be described as a "Rapid Evolution in vitro" hypothesis. This is shown schematically in Table 1. Note that the model predicts how many genes must vary in order to see a given number of intermediate types. As shown in Table 2, a small number of mutating genes would allow a large number of different intermediate strains. Thus mu-

Table 1. Effect of the Number of Variable Properties on the Number of Possible Intermediate Variants of *Bordetella pertussis*

Number of variable properties	1	2	3	4	n
Wild type strain	A	AB	ABC	ABCD	A...Z
				aBCD	
				AbCD	
				ABcD	
				ABCd	
		aBC			
		AbC	abCD		
		ABc	AbcD		
Possible interme-diates			ABcd		
	aB		aBcD		
	Ab		aBCd		
		abC	AbCd		
		Abc			
			abcD		
		aBc	abCd		
			aBcd		
			Abcd		
Final strain	a	ab	abc	abcd	a...z
Number of possible interme-diates	0	2	6	14	$2^n - 2$

Capital letters A, B, C, etc. represent wild type genes. Small letters a, b, c, etc. represent mutants in the corresponding genes. Note that each series is a progression which totals 2^n when the wild type and final strains are included. Thus $2^n - 2$ gives the possible number of intermediate variants.

tations in 4 to 10 genes would allow the possible expression of 14 to 1,022 different phenotypes. Note also that mutations are not necessarily of the "loss" type in this formal model.

Kloos has recently provided evidence that *B. pertussis, B. parapertussis,* and *B. bronchiseptica* have sufficient DNA homology to be considered members of a single species (5). Since *B. parapertussis* and *B. bronchiseptica* grow and behave normally in vitro, this finding supports the theory that changes in only a few genes are sufficient to explain the variability seen in *B. pertussis* cultures.

The salient features of this hypothesis are given below.

Rapid Evolution in Vitro Hypothesis

1. A small number of *B. pertussis* genes (probably 4–10) confer the organism's growth and biological properties.
2. During cultivation in vitro, selection for mutation in these genes (or their regulatory genes) is strong.

Table 2. Effect of the Number of Variable Properties on the Number of Possible Intermediate Forms

Number of Variable Properties	Number of Possible Intermediate Forms
1	0
2	2
3	6
4	14
5	30
6	62
7	126
8	254
9	510
10	1,022
11	2,046
12	4,094
13	8,190
14	16,382
15	32,766
16	65,534
17	131,070
18	262,142
19	524,286
20	1,048,574

3. Some or all of the selective pressure for mutation is due to the presence of inhibitory substances in ordinary agar media.
4. Wild type strains resemble each other, as do final strains.
5. Mutation may occur in any gene of the small number, but no particular order of mutation is found. Thus A, B, C, . . . Z tends to become a, b, c, . . . z only as mutations accumulate.
6. The properties of strains with several mutations are extremely variable, since so many phenotypes may be involved.
7. The number of possible intermediate strains is given by the expression

$$2^n - 2$$

where *n* is the number of genes involved in variation.

This hypothesis adequately explains the observed variability in *B. pertussis* strains grown in vitro. Yet an additional type of variability has been observed in *B. pertussis* by Lacey, who reported a phenomenon he termed "modulation" (6). By changing growth conditions, he was able to induce typical strains (which he termed X-mode strains) to become antigenically distinct, avirulent, and different in a number of biological properties. He called the altered growth habit "C-mode growth." The change in properties was readily reversible by culture on appropriate medium. High

concentrations of magnesium most readily induced the X-mode to C-mode variation, but the variation also was accomplished with sodium or potassium salts. Other parameters, such as temperature of incubation (6) and nutrients (7), have also been shown to induce reversible changes. Recently it has been demonstrated that X-mode cells and C-mode cells differ in their cell membrane proteins (8).

Modulation appears to be a nongenetic change in the properties of *B. pertussis*. Examples of such changes are common in the literature concerning pathogenic bacteria. *Yersinia pestis* and its expression of virulence properties is one example (9). However, analogous situations occur in many eubacteria. Piliation and flagellation are two properties whose expression is modified by cultural conditions. Thus modulation is a phenotypic change induced by environmental changes. It may play an important role in the variability of laboratory grown *B. pertussis* cells, but does not adequately explain the extent of variability seen. Modulation is likely to add one more confusing variable to the overall picture.

Thus my answer to the question, *"Is there something peculiar about the genetics of* B. pertussis?" is *"No."* A straightforward mutation hypothesis accounts for all the genetic changes seen in *B. pertussis*. Phenotypic variation (modulation) also occurs, but is not unique to *B. pertussis*.

The Metabolism of *B. pertussis*

The physiology and metabolism of *B. pertussis* have been studied little since the 1950s. Most of the current knowledge is summarized in a recent review (1) or will be presented at this meeting. In this paper I limit myself to proposing a hypothesis to explain some of the growth properties of *B. pertussis*. The essential points of my hypothesis are given below.

Metabolic Lesion Hypothesis

1. The poor growth and unusual features of metabolism displayed by fresh isolates of *B. pertussis* in vitro are due to slight metabolic differences between this organism and *B. parapertussis* and *B. bronchiseptica*. The differences that seem to prevent the rapid growth of *B. pertussis* are referred to as "lesions."

2. Metabolic lesion(s) do not hinder the growth of *B. pertussis* in its natural environment (the nasopharynx), and may help it to grow there.

3. There is probably only one or a few metabolic lesions.

4. The metabolic lesion(s) may involve energy metabolism, biosynthesis, regulation, or a combination of these areas of metabolism. It is likely that at least one lesion involves regulation.

The actual inhibition of rapid growth could have numerous causes, including the following:

• The accumulation of toxic substances from the environment. In this case, the lesion is likely to involve transport mechanisms. One may imagine the cell as being "too permeable."

• The accumulation of metabolic intermediates in large excess. If this is the case, the lesion is likely to involve regulation of enzyme pathways involved in either biosynthesis or respiration.

• Other possibilities, including the following: marked deficiency in a single enzyme, which can be overcome by overproduction of the enzyme; a defective structural component, which can be overcome by overproduction of other structural components; or production of self-inhibitory substances that are neutralized in vivo but not in vitro.

Mutations to relieve the metabolic lesion(s) are as likely to be regulatory mutants as they are to be structural gene mutants.

Thus my answer to the second question, *"Is there something peculiar about the physiology of* B. pertussis?" is *"Yes."* It is likely that some regulatory mechanism of fresh isolates of *B. pertussis* unexpectedly differs from that usually seen in pathogenic *bacteria*. The organisms grow poorly and are subject to extreme selection during in vitro growth. Mutations that protect the cell from its metabolic lesion(s) ensue, resulting in the rapid appearance of mutant types. Mutants may either be in the genes involved in the lesion(s) or in nonspecific genes. For example, mutants in transport genes could either help keep inhibitory substances out or could help get rid of accumulated intermediary metabolites. Transport gene mutants could result in a phenotype displaying better growth, but would not necessarily occur in the gene specifically involved in the lesion(s).

DISCUSSION

Evidence in a number of reports supports the idea that *B. pertussis* shows some peculiarity in metabolism. However, at this time there is no proof

that the metabolism of the organism has unique features. The most compelling facts available tend to suggest that regulation and membrane proteins are likely to be involved in any metabolic lesion. Adenylate cyclase levels of *B. pertussis* are extremely high (10), arguing that some regulatory mechanism is involved. It is also reported that cyclic AMP levels are high in fresh isolates and low in degraded derivatives of *B. pertussis* (11).

The evidence for membrane involvement in the variability of pertussis strains is equally attractive. Fresh isolates differ from old laboratory strains or C-mode grown cells in their membrane proteins (11). The change in membrane proteins induced by magnesium is freely reversible, but degraded strains appear to have an irreversible loss of proteins. Thus the similarities between these two kinds of cells reflect only a phenotypic similarity, not a genotypic one. Cellular transport, metal uptake, cell membrane structure, secretion of extracellular proteins, and the assembly of surface structures are properties that could be affected by changes in cell membrane proteins.

Studies on the metabolism of *B. pertussis* are increasing, as shown by the papers to be presented at this meeting. We shall hear reports on *B. pertussis* oxygen uptake, cytochrome spectrum, superoxide dismutase, catalase, peroxidase, glutamate transport (12), cyclic AMP levels (11), adenylate cyclase activator (13), and glutamine synthetase (14). We may expect that if research continues, the physiologic basis of *B. pertussis* metabolism will soon be elucidated.

PROPOSALS

I propose changes in pertussis terminology and the establishment of a reference strain depository. I also propose the publication of a manual of standard methods for pertussis research.

Proposal 1: Terminology

There is no evidence to prove that X-mode grown cells and C-mode cells are equivalent to phase I and phase IV cells respectively. Thus I suggest caution in the use of these terms. We lack reference strains or reference antisera to designate pertussis strains as phase I, phase II, phase III, or phase IV with confidence. These terms, therefore, should not be used, or should be carefully defined in each report. The term "degraded strain" has meaning and implications, but no generally accepted degraded reference strain is available. Nor do all workers

agree on what constitutes a degraded strain. Therefore it is not sufficient to state that a strain is degraded. Proof must be offered.

I propose that we develop a terminology. I suggest the use of "fresh isolate" for strains freshly isolated on Bordet-Gengou medium without peptone and maintained on that medium for a minimum number of passages. These strains may be assumed to be phase I, but we lack objective tests to verify this. To designate these strains as phase I is to make an assumption. Vaccine strains and laboratory passaged strains have different properties than do fresh isolates and may be referred to as intermediate strains. It is well to describe such strains as completely as possible. For example, one can say that strain 114 grows on 5% blood agar, is highly immunogenic, produces specific toxins, etc. For degraded strains, we must agree on a definition. Shall our definition be based on growth properties, on biological activities, on serological tests, or on some combination of these? We must come to some generally acceptable agreement as to what is meant by the term "degraded strain" as it applies to *B. pertussis*.

Proposal 2: Reference Strains

To be of optimal value, pertussis research must be reproducible from laboratory to laboratory. One variable that can be eliminated is the variability in strains. I propose that we establish a reference laboratory for *B. pertussis* strains, especially those of interest in research and in vaccine manufacture. Such a facility would allow workers to obtain pertussis strains with properties of interest. It could also encourage the verification of experimental results in several laboratories. I know from experience the difficulty one may have in obtaining specific strains. I would like to have the Tohama strain and its "Phase III" derivative from Japan, but so far I have not been able to get them. I did manage to get the vaccine strain from which Branefors (15) derived her streptomycin resistant mutant, but not the mutant itself. I had difficulty in obtaining fresh clinical isolates of *B. pertussis* until I began to do diagnostic cultures. I believe lack of availability of reference strains, experimental strains, and fresh clinical isolates seriously hinders progress in pertussis research.

Reference antisera for serotyping, agglutination tests, controls in diagnostic serology and fluorescent antibody tests are not readily available. Such sera could be of great value in comparing results from

laboratory to laboratory. We might be able to properly designate phase I and phase IV strains, if suitable antisera were available to qualified researchers.

Reference preparations of purified pertussis components are also greatly needed. Dr. Morse, Dr. Munoz, and Dr. Sato and others have described purified fractions which demonstrate biological activity. The deposit of small samples of such material with a reference laboratory for the use of qualified investigators would stimulate productive research. Reference preparations of agglutinogen 1, 2, 3, and other defined cellular components would be of great value.

Proposal 3: Methods

Standardization of the methods in common use for pertussis research is highly desirable. One man's Bordet-Gengou is *not* that of another man. Cotton plugs, plastic beakers, various brands of peptones, various brands of agar, etc., cause variability in culture results. Variations in procedures abound. A recent monograph has made a useful start in standardizing procedures (16). It is only a beginning, however. Currently, if I cannot repeat someone's work, I do not know whether this failure is due to slight differences in technique, or in reagents, or in strains, or whether it reflects a significant finding. Thus I suggest that this assembly sponsor a committee to be responsible for evaluation of techniques and preparation of a manual of standard pertussis procedures.

I realize that the proposals I have made would require a great deal of time and effort. Funding would have to be obtained. Meetings and correspondence of the individuals involved would require time and effort. Decisions on the best methods and most suitable reference strains will not be easy. Nevertheless, only by such a cooperative effort are we likely to make substantial progress in pertussis research in the near future. We have a responsibility to our communities and to our funding agencies to

proceed effectively in our research. I submit that an international cooperative program such as I propose would have major impact on pertussis research.

ACKNOWLEDGMENT

REFERENCES

1. Parker, C. Role of the genetics and physiology of *Bordetella pertussis* in the production of vaccine and the study of host-parasite relationships in pertussis. Adv. Appl. Microbiol. 20:27–42; 1976.
2. Leslie, P. H.; Gardner, A. D. The phases of *Haemophilus pertussis*. J. Hyg. (Camb.) 31:423–434; 1931.
3. Standfast, A. F. B. The phase I of *Haemophilus pertussis*. J. Gen. Microbiol. 5:531–545; 1951.
4. Rowatt, E. The growth of *Bordetella pertussis*: a review. J. Gen. Microbiol. 17:297–326; 1957.
5. Kloos, W.; Dobrogosz, W.; Ezzell, J.; Kimbro, B. These proceedings.
6. Lacey, B. Antigenic modulation of *Bordetella pertussis*. J. Hyg. (Camb.) 58:57–93; 1960.
7. Pusztai, Z.; Joó, I. Influence of nicotinic acid on the antigenic structure of *Bordetella pertussis*. Ann. Immunol. Hung. 10:63–67; 1967.
8. Parton, R.; Wardlaw, A. Cell-envelope proteins of *Bordetella pertussis*. J. Med. Microbiol. 8:47–57; 1975.
9. Burrows, T.; Bacon, G. V and W antigens in strains of *Pasteurella pseudotuberculosis*. Br. J. Exp. Pathol. 41: 38–44; 1960.
10. Hewlett, E.; Wolff, J. Soluble adenylate cyclase from the culture medium of *Bordetella pertussis*: purification and characterization. J. Bacteriol. 127:890–898; 1976.
11. Wardlaw, A. C.; Parton, R. These proceedings.
12. Dobrogosz, W. J.; Ezzell, J. W.; Kloos, W. E.; Manclark, C. R. These proceedings.
13 Hewlett, E. L.; Underhill, L. H.; Vargo, S. A.; Wolff, J.; Manclark, C. R. These proceedings.
14. Zakharova, M. S.; Amelina, I. P.; Fedotova, N. N. These proceedings.
15. Branefors, P. Transformation of streptomycin-resistance in *Bordetella pertussis*. Acta Pathol. Microbiol. Scand. 62:249–254; 1964.
16 Munoz, J.; Bergman, R. *Bordetella pertussis*: immunological and other biological activities. New York and Basel; Marcel Dekker, Inc., 1977.

DNA-DNA Hybridization, Plasmids, and Genetic Exchange in the Genus *Bordetella*

W. E. Kloos, W. J. Dobrogosz, J. W. Ezzell, B. R. Kimbro, and C. R. Manclark

ABSTRACT

DNA–DNA reassociation reactions indicate a very close genetic relationship between reference strains of *Bordetella pertussis*, *B. parapertussis*, and *B. bronchiseptica*. The hydroxyapatite batch technique of Brenner and coworkers was used to separate single-stranded from reassociated, double-stranded DNA. Relative binding at the optimal criterion of 65° C was 88–94% for reactions between *B. parapertussis* and *B. pertussis* DNA and 72–93% for reactions between *B. bronchiseptica* and *B. pertussis* DNA. There was very little reaction in binding at the stringent temperature of 80° C or in the $T_{m(e)}$ of heteroduplexes between DNAs of these so-called species. Leucine and tryptophan auxotrophs of *B. pertussis* strain BB–185 were transformed by DNA of *B. parapertussis* and *B. bronchiseptica* at frequencies close to homologous values, which also indicates a close genetic relationship among these organisms and raises a question as to the validity of their separate species status.

A large number of *B. pertussis* strains from the FDA culture collection and fresh clinical sources and several *B. parapertussis* and *B. bronchiseptica* strains were screened for the presence of plasmid DNA by agarose gel electrophoresis techniques. Further characterization of plasmids was accomplished with dye-buoyant density and sucrose gradient centrifugation, restriction endonuclease, and electronmicroscopy techniques. Most *B. pertussis* and *B. parapertussis* strains contained a presumed, small cryptic plasmid of approximately 3 megadaltons, whose presence or absence has not yet been related to virulence or state of strain degradation. Of the seven *B. bronchiseptica* strains examined, four carried one or more medium-size to large plasmids (molecular mass range: 8–28 megadaltons) in addition to a small plasmid similar in size to that found in *B. pertussis* and *B. parapertussis*. Restriction endonuclease analyses indicated the presence of one *Eco*RI and no *Bam*HI or *Hind*III recognition sequences in the medium-size and large plasmids of *B. bronchiseptica*. *Hinc*II, *Bgl*I, and *Hae*III recognition sequences were numerous in these plasmids and their digest patterns were useful in distinguishing plasmids of similar electrophoretic mobility derived from different sources.

Genetic exchange studies confirmed the presence of a transformation system in *B. pertussis*. By treating *B. pertussis* phase I strains BB–114 and BB–185 with 200 μg/ml of N-methyl-N'-nitro-N-nitrosoguanidine, followed by growth and replica plating of colonies on various defined agar media differing in amino acid composition, we were able to isolate 39 amino acid auxotrophs. Some of these were further characterized by auxonographic techniques, and blocks in biosynthesis could be tentatively elucidated.

In several preliminary experiments, strain BB–185 mutants Leu–12 and TrpE–5 demonstrated good competence in liquid suspension and on agar with 8–10 hrs of exposure to 1–5 μg of donor DNA per ml in the presence of 0.001 M CaCl$_2$.

INTRODUCTION

Very few genetic or genetically oriented studies of *Bordetella* have been reported in the literature. Phenotypic variation involving a number of different characters, e.g., surface antigens, colony morphology, ability to grow on various media, pigmentation, hemagglutination, biological activities, virulence, antibiotic susceptibility, and membrane proteins has been described, but in most instances the genetic basis of this variation has not been determined (1–5).

Terakado et al. (6) were able to transfer resistance to streptomycin, sulfadimethoxine, and aminobenzyl penicillin in several *Bordetella bronchiseptica* strains as one unit to a susceptible strain of *Escherichia coli* or *B. bronchiseptica* by mixed cultivation. They were also able to eliminate the acquired triple resistance in suspected exconjugants, by growing them in the presence of acriflavin. Later Hedges et al. (7) confirmed the presence of an R plasmid in one of the triple-resistant *B. bronchiseptica* strains above and determined its molecular mass to be 34.6 megadaltons. These studies suggested that *B. bronchiseptica*, like many other bacteria, carry certain antibiotic resistance genes on plasmids and that this organism may par-

ticipate in a conjugation or a conjugationlike process.

Branefors (8) was able to transform a streptomycin-susceptible strain of *Bordetella pertussis* to streptomycin resistance by exposure to isolated DNA from a streptomycin-resistant mutant of the same strain, thereby demonstrating for the first time the presence of a transforming system in this species.

Though transduction has not yet been reported in *Bordetella*, bacteriophages have been isolated from *B. bronchiseptica* (9) and *B. pertussis* (S. Mebel and I. Lapaeva, unpublished observations) which, in the future, may be explored for transducing activity.

This report will give some new basic information on the genetic relationships of various *Bordetella*, characterization of *Bordetella* plasmids, and transformation in *B. pertussis*.

SYSTEMATICS OF THE GENUS
Bordetella

Division of *Bordetella* on the Basis of Phenotype

Currently, the genus *Bordetella* is composed of three species, *B. pertussis* (the type species), *B. parapertussis*, and *B. bronchiseptica* (10). Species can be distinguished on the basis of several key phenotypic characters as shown in Table 1.

The distinction between *B. pertussis* and *B. parapertussis* is not always clear, but usually *B. parapertussis* grows well on blood-free peptone agar and produces a browning of this medium (pigment from tyrosine), utilizes citrate, produces urease, grows more rapidly on Bordet-Gengou (BG) agar, and possesses the specific heat-labile antigen, Factor 14. Some "degraded" or phase IV strains of *B. per-*

tussis can also grow well on blood-free peptone agar and BG agar.

Recently, Kumazawa and Yoshikawa (11) described experiments converting a phase I strain of *B. pertussis* to mutants resembling *B. parapertussis* with the use of N-methyl-N'-nitro-nitrosoguanidine (NTG). Like *B. parapertussis*, the resulting pleiotropic mutants produced black pigmentation on BG agar and brown pigmentation of nutrient agar supplemented with tyrosine. Most also become more resistant to certain antibiotics, such as nalidixic acid, streptomycin, penicillin G, and ampicillin; had low dermonecrotic activity; produced relatively large colonies on BG agar; and utilized citrate like typical *B. parapertussis* strains. Some demonstrated intermediate characteristics.

In our independent studies, with the use of NTG, we have also produced mutants of *B. pertussis* that resemble *B. parapertussis* with regard to brown pigmentation and colony morphology. It is also rather interesting that many *B. parapertussis* strains may serve as indicator strains for *B. pertussis* (S. Mebel and I. Lapaeva, unpublished observations) and *B. bronchiseptica* bacteriophages (9). The close phenetic relationship of *B. pertussis* and *B. parapertussis* strains studied by Johnson and Sneath (12) was exemplified by their average intergroup similarity of 85%. On the basis of these recent observations, we might begin to question the validity of giving *B. pertussis* and *B. parapertussis* separate species ranking.

B. bronchiseptica can be distinguished from other *Bordetella* primarily on the basis of rapid growth on blood-free peptone agar, motility, nitrate reduction, early urease activity, and the presence of a specific heat-labile antigen factor 12. This species has been isolated from a variety of animal species, in contrast to *B. pertussis* and *B. parapertussis*,

Table 1. Distinguishing Phenotypic Characters of Species of the Genus *Bordetella*

Character	*B. pertussis*	*B. parapertussis*	*B. bronchiseptica*
Growth on Bordet-Gengou agar	Slow; small colonies	Moderate; slightly larger colonies	Moderate to rapid; larger colonies
Growth on blood-free peptone agar	Phase I — Phase IV +	+ With browning	+
Motility	—	—	+
Citrate utilization	—	±, +	+
Urease activity	—	+	rapid +
Nitrate reduction	—	—	+
Specific heat-labile antigen (Factor)	1	14	12

Reactions or response: +, positive; ±, weak positive; —, negative

which are predominantly isolated from humans. The numerical taxonomic study of Johnson and Sneath (12) indicated an average intergroup similarity of approximately 80% between *B. bronchiseptica* and the other two species.

Evidence for One DNA Homology Group Within the Genus *Bordetella*

To provide information on the genetic relationships of the *Bordetella* species, we performed DNA–DNA reassociation reactions using ^{32}P-labeled *B. pertussis* DNA with unlabeled *B. pertussis*, *B. parapertussis*, or *B. bronchiseptica* DNA. The reactions were carried out in duplicate using 0.1μg of sheared, heat denatured, ^{32}P-labeled DNA and 120μg of unlabeled DNA in 1 ml of 0.28M phosphate buffer with incubation at either 65° or 80° C for 20 hours. The hydroxyapatite batch technique of Brenner and coworkers (13) was used to separate single-stranded from reassociated, double-stranded DNA. The results are shown in Tables 2 and 3. As can be seen from the data, relative binding at the optimal criterion of 65° C was 88–94% for reactions between *B. pertussis* and *B. parapertussis* DNA and 72–93% for reactions between *B. pertussis* and *B. bronchiseptica* DNA. There was very little or no reduction in binding at the stringent temperature of 80° C and only a slight reduction in the $T_{m(e)}$ of heteroduplexes between DNAs of these so-called species. These preliminary findings strongly suggest that strains identified as typical *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* have not diverged enough genetically to be classed as separate species. Furthermore, we have been able to demonstrate that selected leucine and tryptophan auxotrophs of *B. pertussis* strain BB–185 can be

Table 2. DNA–DNA Reassociation Reactions Using ^{32}P-Labeled DNA from *Bordetella pertussis* Strain BB–114 (Phase I)

Source of Unlabeled DNA	Average Relative Binding at 65°C	Average Relative Binding at 80°C	$\Delta T_{m(e)}$ at 65°C (% divergence)
B. pertussis BB–114(I)	100	100	
B. pertussis L51 (Dolby) (IV)	106	95	−0.7
B. parapertussis 21449	94	76	−1.2
B. bronchiseptica D–2 (B)	93	79	−1.6
E. coli K12	8	4	ND

ND, not determined

Table 3. DNA–DNA Reassociation Reactions Using ^{32}P-Labeled DNA from *Bordetella pertussis* Strain L51 (Dolby) (Phase IV)

Source of Unlabeled DNA	Average Relative Binding at 65°C	Average Relative Binding at 80°C	$\Delta T_{m(e)}$ at 65°C (% divergence)
B. pertussis L51 (Dolby) IV	100	100	
B. pertussis BB–114 (I)	103	98	−0.3
B. parapertussis 21449	88	88	−0.7
B. bronchiseptica D–2 (B)	72	106–111[a]	−0.9
E. coli K12	9	6	ND

[a] This unusually high relative binding at 80°C was repeatable in the hands of two of us (W. E. K. and J. W. E.), but is as yet unexplained.

ND, not determined

transformed by DNA of *B. parapertussis* and *B. bronchiseptica* to independence of these amino acids at frequencies rather close to homologous values, providing additional proof of a close genetic relationship among these organisms. Genetic transfer between various *Bordetella* may offer the possibility of selecting or constructing strains possessing a combination of optimal features for vaccine production.

Bordetella PLASMIDS

Occurrence and Preliminary Characterization of Different Plasmids

Forty-three *B. pertussis* strains from the FDA culture collection; 23 *B. pertussis* isolates from various fresh clinical sources in Atlanta and Decatur, Georgia, St. Louis, Missouri, and Austin, Texas; two *B. parapertussis*, and seven *B. bronchiseptica* strains were screened for the presence of plasmid DNA using agarose gel electrophoresis techniques (14, 15). Further characterization of plasmids was accomplished with dye-buoyant density gradient centrifugation, rate zonal centrifugation in neutral sucrose gradients, restriction endonuclease, and electronmicroscopy techniques (16–19).

Most *B. pertussis* and both *B. parapertussis* strains contained only a small cryptic plasmid with a mass of approximately 3 megadaltons (Fig. 1). So far, we have not been able to relate the presence or absence of this plasmid to virulence or state of strain degradation. Furthermore, we cannot be sure at this point that the *B. pertussis* strains failing to show this plasmid do not actually contain it.
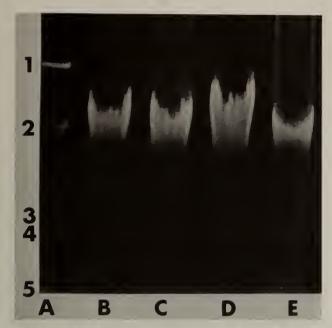
Figure 1. Agarose gel electrophoresis of *Bordetella pertussis* DNA. Plasmid DNA was preferentially isolated by techniques involving the shearing and alkaline denaturation of chromosomal DNA, followed by preferential removal of single-stranded DNA by phenol extraction (14). Plasmid DNA was then precipitated by treatment with cold 95% ethanol and magnesium phosphate precipitate and dissolved in 0.1M EDTA, pH 8.0. The concentrated DNA was dialyzed against TES buffer (0.05M Tris, 5mM EDTA, 0.05M NaCl, pH 8.0) and 50μl of sample were applied to 0.7% agarose gels (15). Migration was from top (cathode) to bottom (anode). Electrophoresis was carried out in a vertical slab cell at 180 volts and 60mA for approximately 2 hours. Gels were placed in a solution of ethidium bromide (0.4μg/ml of distilled H_2O), stained for 15 to 30 minutes, and then observed with long wave ultraviolet light. (A) *Escherichia coli* reference plasmids; 1, Sa (molecular weight, 23×10^6); 2, chromosomal fragments; 3 (very faint), pMB8 dimer (molecular weight, 3.74×10^6); 5, pMB8 (molecular weight, 1.8×10^6). (B) *B. pertussis* strain A4785; 2, chromosomal fragments; 4 (very faint), small suspected plasmid. (C) *B. pertussis* strain 77–106325; 2, chromosomal fragments; 4, small suspected plasmid. (D) *B. pertussis* strain L–4 (Sato) (phase I); 2, chromosomal fragments; 4, small suspected plasmid. (E) *B. pertussis* strain BB–114; 2, chromosomal fragments; 4, small suspected plasmid.

This is because we must overload agarose gels with isolated DNA (chromosomal plus enriched plasmid DNA) to observe a faint fluorescent plasmid band. Some strains could conceivably have a concentration of this DNA just below visible detection. Evidence indicating that the DNA in this faint band is plasmid DNA is incomplete, as we have not yet been able to isolate it in sufficient quantity for restriction endonuclease analyses or EM photography. It can be originally isolated in very small quantity by dye-buoyant density gradient centrifugation (Fig. 2) and then observed by agarose gel
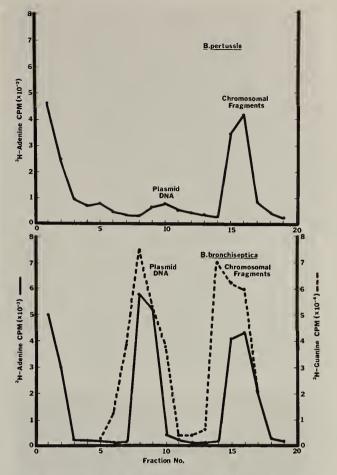

Figure 2. Equilibrium CsCl-EthBr density gradient centrifugation of *Bordetella pertussis* and *Bordetella bronchiseptica* DNA. Cultures of *B. pertussis* strain 77–106325 were grown in the presence of 1μCi ^{3}H-adenine/ml in 500 ml of modified Stainer-Scholte broth medium (22) for 16 hours at 34°C, then harvested. Cultures of *B. bronchiseptica* strain D–1 (ATCC 31124) were grown in the presence of 1μCi ^{3}H-adenine or ^{3}H-guanine/ml in 500 ml of Brain Heart Infusion broth for 5 hours and then harvested. DNA was isolated according to the procedures of Currier and Nester (14). Gradients were formed by centrifugation at 125,000 × g for 42 hours in a fixed angle type 65 rotor. Ten-drop fractions were collected from the bottom of the tube and 0.02 ml samples of each fraction were removed and counted. The peak of radioactivity at the bottom of the tube most probably represents RNA. The *Bordetella* strains failed to incorporate adequate levels of [methyl-^{3}H] thymidine into DNA for more specific labeling.

electrophoresis, but any attempts to concentrate it further have failed. This presumed plasmid may be very sensitive to contaminating nucleases, though consistent with its identity as DNA it is digested by bovine pancreatic ribonuclease.

Four of the seven *B. bronchiseptica* strains examined carried one or more medium-size to large plasmids, in addition to a small labile plasmid similar in size and concentration to that found in

B. pertussis and *B. parapertussis* (Figs. 2, 3). The common carriage of relatively large plasmids in *B. bronchiseptica* was also inferred earlier by Terakado et al. (6) in their studies of R factors in *B. bronchiseptica* isolated from pigs.

More detailed characterization of several of the *B. bronchiseptica* plasmids led to some interesting observations to be discussed below. For the characterization we selected plasmids from strain D–1 (ATCC 31124) isolated from a dog suffering from distemper; D–2 (B), a canine virulent isolate; and D–2 (D), a reisolated culture of D–2 (B) passed in another dog, which were all supplied by Robert A. Goodnow (Burns-Biotec, Omaha, Nebraska).

Strain ATCC 31124 contained one medium-size plasmid, designated pWE100, with a molecular mass of 8.2 megadaltons [based on contour length (CL)] to 8.27 megadaltons [based on restriction endonuclease analyses (RE)] and one relatively large plasmid, designated pWE101, with a molecular mass of 23.0 (CL) to 24.5 (RE) megadaltons. These plasmids were first separated from chromosomal DNA and isolated together in their co-
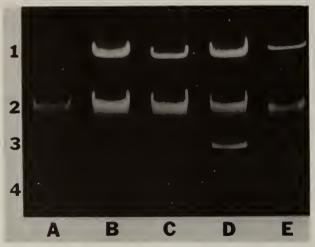
valently closed circular form (CCC) from a cesium chloride gradient containing ethidium bromide, then separated from each other in a neutral sucrose gradient containing a very low concentration of ethidium bromide. Dialyzed samples of plasmids were examined during each step of isolation by agarose gel electrophoresis to check purity (Fig. 4) and further examined by electronmicroscopy (Fig. 5).

Strain D–2 (B) and the reisolated culture D–2 (D) both appear to possess one large plasmid, designated pWE102, with a molecular mass of 25.0 (CL) to 26.9 (RE) megadaltons.

Restriction Endonuclease Analysis of Selected *B. bronchiseptica* Plasmids

Since both pWE101 and pWE102 plasmids migrated to similar positions in agarose gels during
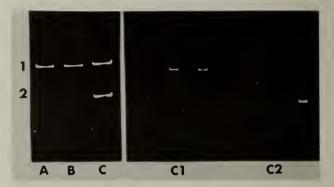

Figure 4. Agarose gel electrophoresis of isolated *B. bronchiseptica* plasmid DNA. Plasmid DNA was preferentially isolated and concentrated by techniques described by Currier and Nester (14) and further separated from chromosomal DNA by equilibrium CsCl-EthBr density gradient centrifugation. Gradients were formed by centrifugation at 125,000 × g for 44 hours in a fixed angle type 65 rotor. The more dense fluorescent band containing CCC plasmid DNA was collected from the bottom of the tube and ethidium bromide was removed by extraction with isopropanol saturated with CsCl. Extracted samples were dialyzed for 2 days against TES buffer and then checked for purity by agarose gel electrophoresis (left panel). (A) 1, plasmid pWE102 from strain D–2 (B). (B) 1, Plasmid pWE102 from strain D–2 (re-isolate D). (C) 1, plasmid pWE101; 2, plasmid pWE100 from strain D–1 (ATCC 31124). Dialyzed strain ATCC 31124 plasmid DNA was layered onto 17 ml 5–20% neutral sucrose gradients in the presence of 4μg of ethidium bromide per ml (17). Good separation of plasmid pWE101 from pWE100 was obtained by centrifugation at 94,500 × g for 4 hours in a swinging bucket rotor AH627. The well-separated and visible plasmid bands were collected separately from the bottom of the tube and ethidium bromide was removed by dialysis against TES buffer. Samples were checked for purity by agarose gel electrophoresis (right panel). (C1) purified plasmid pWE101; from left to right 10μl, 20μl and 30μl samples. (C2) purified plasmid pWE100; from left to right 10μl, 20μl, and 60μl samples. Migration was from top (cathode) to bottom (anode). Electrophoresis conditions were the same as described in Figure 1.

Figure 3. Agarose gel electrophoresis of *Bordetella bronchiseptica* DNA. Plasmid DNA was preferentially isolated and concentrated by techniques described by Currier and Nester (14). Twenty μl of DNA sample were applied to 0.7% agarose gels (15). Migration was from top (cathode) to bottom (anode). Electrophoresis conditions were the same as described in Figure 1. (A) *B. bronchiseptica* strain C; 2, chromosomal fragments. (B) *B. bronchiseptica* strain D–2 (B); 1, pWE102; 2, chromosomal fragments. (C) strain D–2 (re-isolate D); 1, pWE102; 2, chromosomal fragments. (D) *B. bronchiseptica* strain D–1 (ATCC 31124); 1, pWE101; 2, chromosomal fragments; 3, pWE100. (E) *Escherichia coli* reference plasmids; 1, Sa (molecular weight, 23 × 10⁶); 2, chromosomal fragments; 4 (very faint), pMB8 dimer (molecular weight, 3.74 × 10⁶). Small suspected plasmids forming a very faint fluorescent band below position 4 were observed with each *B. bronchiseptica* strain, but these were not reproduced adequately in this photograph.
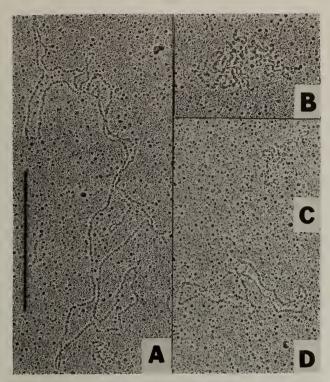
Figure 5. Electron micrograph of *Bordetella bronchiseptica* pWE101 and pW100 plasmids. Bar equals 1 μm. (A) pWE101, open circular (OC) form; average molecular weight, 23.0 × 10⁶. (B) pWE101, native covalently closed circular (CCC) form. (C) pWE100, native CCC form. (D) pWE100, OC form; average molecular weight, 8.2 × 10⁶.

Figure 6. *Hinc*II fingerprints of *Bordetella bronchiseptica* plasmids. Purified plasmid and reference bacteriophage λ DNA were digested with *Hinc*II restriction endonuclease, according to recommendations supplied by the vendor, New England Biolabs, Beverly, Massachusetts. Migration in a 1.0% agarose gel was from top (cathode) to bottom (anode). Conditions for electrophoresis have been described in Figure 1. (A) Digest DNA fragments of plasmid pWE102, isolated from *B. bronchiseptica* strain D–2(B). (B) Fragments of plasmid pWE102 isolated from the reisolated culture D–2 (D), obtained after passage of D–2 (B) through another dog. (C) Fragments of plasmid pWE101 isolated from *B. bronchiseptica* strain D–1 (ATCC 31124). (D) Digest DNA of plasmid pWE100 from strain ATCC 31124. (E) Fragments of bacteriophage λ. These and fragments of φ X 174 RF DNA-*Hinc*II digest (not shown in photograph) were used as molecular weight reference markers. An approximate molecular weight scale is at the right margin of the figure. Fragments are numbered consecutively from top (largest) to bottom (smallest). The number of fragments indicated for each band is tentative and was estimated by measuring the relative intensity of band fluorescence, taking into account the expected reduction in intensity with decreasing molecular size. The very faint bands occurring above Fragment 1 in two of the digests most probably represent very low concentrations of partially-digested fragments. They were not observed in four subsequently replicated digests and their absence did not alter the numbered fragment pattern shown here.

electrophoresis (Figs. 3, 4), we wondered if they were identical. In addition, we wished to examine the pWE101 plasmids from strain D–2 (B) and its reisolate D–2 (D) to see if subculture of the host strain in a different dog affected its plasmid structure or composition. To answer these inquiries, we chose to characterize each plasmid, using different restriction endonuclease enzymes to estimate the number and position of certain recognition (nucleotide) sequences in the plasmid genome (18, 19). The recognition sequences, specific for different enzymes, may serve as markers for fingerprinting plasmids, especially if their number in the genome is relatively large.

We were able to detect only one *Eco*RI and no *Bam*HI or *Hind*III recognition sequences in these *B. bronchiseptica* plasmids. However, *Hinc*II, *Hae*II, *Bgl*I, and *Hae*III recognition sequences were numerous in the relatively large plasmids, pWE101 and pWE102. *Hinc*II (Fig. 6), *Hae*II (Fig. 7), and *Bgl*I (not shown) digest patterns were very useful in distinguishing these two plasmids of similar electrophoretic mobility derived from different sources. The digest patterns for plasmid pWE102
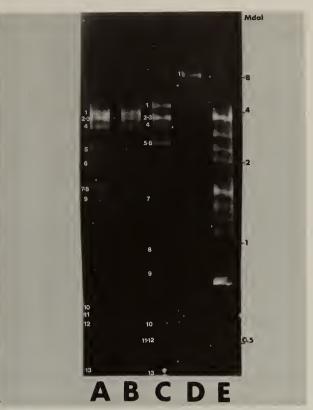
from strain D–2 (B) and its reisolate D–2 (D) were identical. *Hae*III digests contained only very small DNA fragments (<0.4 megadalton) which were too numerous and difficult to resolve.

Of the several different restriction endonuclease

Figure 7. *Hae*II fingerprints of *Bordetella bronchiseptica* plasmids. Purified plasmid and reference bacteriophage λ DNA were digested with *Hae*II restriction endonuclease according to recommendations supplied by the vendor, New England Biolabs, Beverly, Massachusetts. Migration in a 1.4% agarose gel was from top (cathode) to bottom (anode). Conditions for electrophoresis have been described in Figure 1. (A′) Undigested DNA and (A) digested DNA fragments of plasmid pWE102, isolated from *B. bronchiseptica* strain D–2 (B). (B′) Undigested DNA and (B) digested fragments of plasmid pWE102, isolated from the reisolated culture D–2 (D), obtained after passage of D–2 (B) through another dog. (C′) Undigested DNA and (C) digested fragments of plasmid pWE101, isolated from *B. bronchiseptica* strain D–1 (ATCC 31124). (D′) Undigested DNA and (D) digested fragments of plasmid pWE100 from strain ATCC 31124. (E) Fragments of bacteriophage λ. These and fragments of φ X 174RF DNA-*Hinc*II digest (not shown in photograph) were used as molecular weight reference markers. An approximate molecular weight scale is at the right margin of the figure. Fragments are numbered consecutively from top (largest) to bottom (smallest). The number of fragments indicated for each band is tentative and was estimated by measuring the relative intensity of band fluorescence, taking into account the expected reduction in intensity with decreasing molecular size. Identical fragment patterns were observed for four other replicated digests.

enzymes tested, we conclude that *Hinc*II, *Hae*II, and *Bgl*I would be the most useful to be applied to *Bordetella* plasmid epidemiological studies. We have found that most of the DNA fragments generated by these enzymes are easily separated on agarose or polyacrylamide-agarose gels (15, 20). *Hinc*II cleaved each of the pWE101 and pWE102 plasmids into at least 13 fragments and the smaller

pWE100 plasmid only once (Fig. 6). Although most of the DNA fragments produced from the pWE101 and pWE102 plasmids are different, Fragments 2, 3, and 4 in both plasmids may be identical; and Fragment 9 in pWE102 may be common to Fragment 7 in pWE101. Further analyses, for example, involving DNA–DNA hybridization of the specific fragments would be necessary to prove identity. We have recently determined that the single *Eco*RI recognition sequence in plasmid pWE102 is located in *Hinc*II Fragment 7 or 8.

*Hae*II cleaved each of the pWE101 and pWE102 plasmids into more than 30 fragments and the smaller pWE100 plasmid into at least 16 fragments (Fig. 7). Fragments 4, 5, and 12 or 13 in pWE102 may be identical with Fragments 6, 7, and 12, respectively, in pWE101. Some of the fragments numbered 7 through 10 in pWE102 may be identical with certain of the fragments numbered 8 through 10 in pWE101 and numbers 1 and 2 in pWE100. There may also be identity of some of the fragments numbered 18 through 20 in pWE102 with fragments numbered 16 through 18 in pWE101 and numbers 6 and 7 in pWE100. These results suggest that there may be sequences conserved or shared between some of the *B. bronchiseptica* plasmids, reflecting, in part, a common origin.

*Bbl*I cleaved each of the pWE101 and pWE102 plasmids into more than 40 fragments and the smaller pWE100 plasmid into at least 18 fragments. There was an unusually large number of fragments (8–10) of equal size (0.42 megadalton) cleaved from plasmid pWE100. They appeared together as a very bright band on agarose gels; other fragments produced much fainter bands by comparison.

In the future, it should be possible to isolate specific fragments of *B. bronchiseptica* plasmids and begin to determine their genetic function. If selective markers are found, it should also be possible to construct plasmid chimeras containing *Bordetella* DNA that may have particular use in cloning experiments.

TRANSFORMATION IN *B. pertussis*

Development of a Defined Agar Medium for the Isolation of Amino Acid Auxotrophs

As mentioned earlier above, Branefors (8) was able to demonstrate transformation of an antibiotic resistance marker in *B. pertussis*. This stands as an isolated report. We were interested in extending transformation studies to include genes associated with amino acid biosynthesis to begin fine structure

slow growth and therefore was not particularly desirable for genetic studies.

In one experiment, by treating *B. pertussis* phase I strains BB–114 and BB–185 with 200μg of NTG per ml, we were able to isolate 39 amino acid auxotrophs. In this experiment we specifically selected tryptophan, leucine, isoleucine, and isoleucine-valine auxotrophs by replica plating the colonies of mutagenized cells onto a series of defined media lacking one of these amino acids at a time. Auxotrophs could be distinguished as early as 24 hours after replica plating by their very poor relative growth response.

Characterization of Amino Acid Auxotrophs

Of the total amino acid auxotrophs originally isolated, 10 leucine, 5 isoleucine, 4 tryptophan, and 1 isoleucine-valine mutant of strain BB–185 and 1 leucine, 3 isoleucine, and 1 tryptophan mutant of strain BB–114 exhibited low or no detectable reversion rate. These auxotrophic mutants were then characterized by auxonographic techniques to initially estimate the enzyme block(s) and gene(s) affected in the respective biosynthetic pathways.

Most leucine mutants proved to be too leaky for accurate interpretation of growth response to various biosynthetic pathway intermediates. The leaky background growth produced by these mutants also interfered with the expression of recombinants in genetic transfer experiments, so they were omitted from further study. The growth responses of two nonleaky leucine mutants of strain BB–185, Leu–12 and Leu–15, are shown in Figure 8. Both mutants grew in the presence of either L-leucine or α-keto-isocaproate, suggesting that they were blocked at a step prior to the transamination of α-ketoisoca-proate to L-leucine (23).

Three of the four BB–185 tryptophan mutants, TrpE–2, TrpE–3, and TrpE–5, grew in the presence of either L-tryptophan, anthranilic acid, or indole (Fig. 8), suggesting that they had no or very reduced anthranilate synthetase activity (block at the first step in tryptophan biosynthesis) (24). BB–185 Trp–4 grew in the presence of L-tryptophan or indole; BB–114 TrpB–6 grew only in the presence of L-tryptophan and is presumably blocked in the last step of tryptophan biosynthesis.

Four BB–185 isoleucine mutants, IleA–1, IleA–2, IleA–3, and IleA–4, and two BB–114 isoleucine mutants IleA–12 and IleA–13 grew in the presence of L-isoleucine, α-keto-β-methylvaleric acid, and α-keto-isovaleric acid, or α-ketobutyrate and pyru-

vate (kindly supplied by Frank B. Armstrong, North Carolina State University, Raleigh, N.C.), suggesting that they had no or very reduced threonine deaminase activity (block at first step in isoleucine biosynthesis) (25). One BB–185 isoleucine-valine mutant, IlvaC–6, grew only in the presence of L-
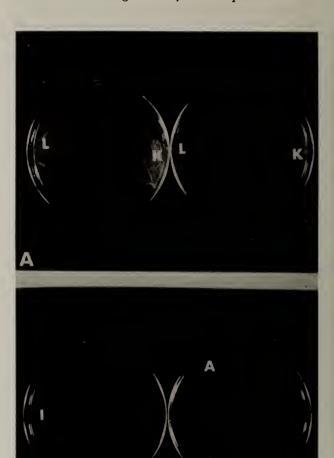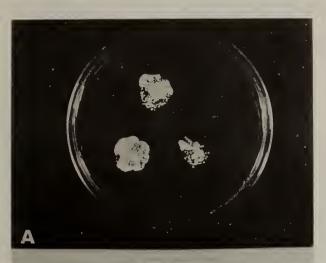
Figure 8. **Growth of *Bordetella pertussis* strain BB–185 leucine and tryptophan auxotrophs in the presence of required amino acids and their biosynthetic pathway intermediates.** Approximately 10^7 colony-forming units of each auxotroph were spread over the surface of a defined agar medium lacking either L-leucine or L-tryptophan. (A) Several crystals of L-leucine (L) or a solution of the L-leucine pathway intermediate α-ketoisocaproate (K) were placed at widely separated points on the surface of agar inoculated with the leucine mutants, Leu-12 (left) and Leu-15 (right). (B) Several crystals of L-tryptophan (T) or the L-tryptophan pathway intermediates anthranilic acid (A) or indole (I) were placed at widely separated points on the surface of the same or different agar plates inoculated with the tryptophan mutant TrpE–3. Plates were incubated at 34° C for 48 hours and then examined for evidence of growth in the region of each metabolite.

isoleucine and L-valine and is presumably blocked in the last step (transaminase) of isoleucine and valine biosynthesis. Enzymes catalyzing the various steps in biosynthesis will have to be assayed in the various mutants described above to confirm suspected blocks and identify specific genes affected by mutation.

Transformation of Amino Acid Auxotrophs

Three of the above auxotrophic mutants, BB–185 TrpE–5, BB–185 Leu–12, and BB–114 IleA–12, were selected to serve as recipients in transformation experiments. In several preliminary experiments, the two BB–185 mutants demonstrated good competence in liquid suspension and on agar with 8–10 hours exposure to 1 to 5μg of donor parent BB–185 DNA per ml in the presence of 0.001M CaCl$_2$. Tryptophan- and leucine-independent recombinant colonies derived by transformation of mutant recipient cells with donor DNA in liquid suspension or by application of donor DNA onto the surface of agar plates inoculated with mutant recipient cells are shown in Figure 9. Transformants could be detected as early as 72–96 hours. The BB–114 IleA–12 mutant demonstrated a very low level of competence in one experiment. In two other attempts, we were unable to demonstrate transformation of this mutant. We are now determining optimal conditions for transformation. Our studies have clearly confirmed the presence of a transformation system in *B. pertussis* strain BB–185, but additional information will be required to bring the frequency of transformation up to a level that will be useful in fine structure mapping. Furthermore, although transformation was detectable with several freshly isolated auxotrophs, upon additional subculture these auxotrophs lost much of their competency. We are currently attempting to isolate auxotrophs demonstrating stable competency in transformation.

Figure 9. Transformant colonies of *Bordetella pertussis* strain **BB–185**. (A) Tryptophan-independent recombination colonies appearing in the area where a drop of donor parent (Trp+) DNA (10 to 20 μg/ml) was applied to the surface of a defined amino acid, agar medium spread with approximately 5 × 10⁷ colony-forming units of the recipient mutant TrpE–5. The agar medium lacked L-tryptophan. (A) 0.1 ml aliquot of a 0.01M CaCl$_2$ solution was applied to the surface of the agar and spread with a hockey stick until absorbed, prior to inoculation of the agar surface with recipient cells. Plates treated with DNA were incubated for 4 days at 34° C, then examined for the presence of colonies or growth. (B) Leucine-independent recombination colonies appearing on a defined amino acid, agar medium spread with approximately 5 × 10⁷ colony-forming units of the recipient mutant Leu-12 previously exposed to 5μg of donor parent (Leu+) DNA per ml for 10 hours at 34° C. Exposure to donor DNA took place in a modified Stainer-Scholte broth medium (22) containing 0.25% casein hydrolysate (enzymatic digest) and 0.001M CaCl$_2$. The agar medium lacked L-leucine. Inoculated plates were incubated for 4 days at 34° C and the resulting colonies were enumerated with the aid of a Quebec colony counter.

REFERENCES

1. Leslie, P. H.; Gardner, A. D. The phases of *Haemophilus pertussis*. J. Hyg. (Camb.) 31:423–434; 1931.
2. Lacey, B. W. Antigenic modulation of *Bordetella pertussis*. J. Hyg. (Camb.) 58:57–93; 1960.
3. Wardlaw, A. C.; Parton, R.; Hooker, M. J. Loss of protective antigen, histamine sensitizing factor and envelope

polypeptides in cultural variants of *Bordetella pertussis*. J. Med. Microbiol. 9:89–100; 1976.

4. Parker, C. Role of the genetics and physiology of *Bordetella pertussis* in the production of vaccine and the study of host-parasite relationships in pertussis. Adv. Appl. Microbiol. 20:27–42; 1976.

5. Cameron, J. Problems associated with the control testing of pertussis vaccine. Adv. Appl. Microbiol. 20:57–80; 1976.

6. Terakado, N.; Azechi, H.; Ninomiya, K.; Shimizu, T. Demonstration of R factors in *Bordetella bronchiseptica* isolated from pigs. Antimicrob. Agents Chemother. 3:555–558; 1973.

7. Hedges, R. W.; Jacob, A. E.; Smith, J. T. Properties of an R factor from *Bordetella bronchiseptica*. J. Gen. Microbiol. 84:199–204; 1974.

8. Branefors, P. Transformation of streptomycin-resistance in *Bordetella pertussis*. Acta. Path. et Microbiol. Scand. 62:249–254; 1964.

9. Rauch, H. C.; Pickett, M. J. *Bordetella bronchiseptica* bacteriophage. Can. J. Microbiol. 7:125–133; 1961.

10. Pittman, M. Genus *Bordetella* Moreno-Lopez 1952. Buchanan, R. E.; Gibbons, N. E., eds. Bergey's manual of determinative bacteriology, 8th edition. Baltimore: Williams and Wilkins Co.; 1974: 282–283.

11. Kumazawa, N.; Yoshikawa, M. Conversion of *Bordetella pertussis* to *Bordetella parapertussis*. J. Hyg. (Camb.) 81:15–23; 1978.

12. Johnson, R.; Sneath, P. H. A. Taxonomy of *Bordetella* and related organisms of the families. *Achromobacteraceae, Brucellaceae, and Neisseriaceae*. Int. J. Syst. Bacteriol. 23:381–404; 1973.

13. Brenner, D. J.; Fanning, G. R.; Rake, A. V.; Johnson, K. E. Batch procedure for thermal elution of DNA from hydroxyapatite. Anal. Biochem. 28:447–459; 1969.

14. Currier, T. C.; Nester, E. W. Isolation of covalently closed circular DNA of high molecular weight from bacteria. Anal. Biochem. 76:431–441; 1976.

15. Meyers, J. A.; Sanchez, D.; Elwell, L. P.; Falkow, S. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. 127:1529–1537; 1976.

16. Bauer, W.; Vinograd, J. The interaction of closed circular DNA with intercalative dyes. 1. The superhelix density of SV40 DNA in the presence and absence of dye. J. Mol. Biol. 33:141–171; 1968.

17. Hugh, C.; Meynell, G. G. Rapid screening for plasmid DNA. Molec. Gen. Genet. 151:175–179; 1977.

18. Sadowski, P. L.; Peterson, B. C.; Gerding, D.; Cleary, P. P. Use of a restriction endonuclease to establish the persistence and epidemic spread of a single R plasmid in the hospital environment. Schlessinger, D., ed. Microbiology—1978. Washington, D.C.: American Society for Microbiology; 1978: 270–272.

19. Sciaky, D.; Montoya, A.; Chilton, M. D. Fingerprints of *Agrobacterium* Ti plasmids. Plasmid 1:238–253; 1978.

20. Peacock, A. C.; Dingman, C. W. Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose–acrylamide composite gels. Biochemistry 7:668–674; 1968.

21. Stainer, D. W.; Scholte, M. J. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. J. Gen. Microbiol. 63:211–220; 1971.

22. Hewlett, E.; Wolff, J. Soluble adenylate cyclase from the culture medium of *Bordetella pertussis*. Purification and characterization. J. Bacteriol. 127:890–898; 1976.

23. Umbarger, E.; Davis, B. D. Pathways of amino acid biosynthesis. Gunsalus, I. C.; Stanier, R. Y., eds. The bacteria, vol. 3. New York: Academic Press, Inc.; 1962: 167–252.

24. Smith, O. H.; Yanofsky, C. Enzymes involved in the biosynthesis of tryptophan. Colowick, S. P.; Kaplan, N. O., eds. Methods in enzymology, vol. 5. New York: Academic Press, Inc.; 1962: 794–806.

25. Wagner, R. P.; Bergquist, A. Nature of genetic blocks in the isoleucine-valine mutants of Salmonella. Genetics 45:1375–1386; 1960.

Bordetella pertussis Adenylate Cyclase: Regulation of Activity and Its Loss in Degraded Strains

E. L. Hewlett, L. H. Underhill, S. A. Vargo, J. Wolff, and C. R. Manclark

ABSTRACT

Bordetella pertussis possesses an extracytoplasmic adenylate cyclase, a substantial proportion (approximately 40%) of which is measurable when intact organisms are assayed using exogenous ^{32}P–ATP as substrate. The activity of this whole cell-associated enzyme is increased markedly by a protein factor from erythrocytes. When organisms are cultured on Bordet-Gengou (BG) agar containing rabbit blood, the adenylate cyclase activity is high (10–100 nmol cAMP formed/min/mg protein) and unaffected by exposure to additional activator. When these organisms are transferred to Stainer-Scholte (SS) liquid medium without blood, there is a decrease in adenylate cyclase activity over the first hour, followed by a gradual increase in activity with growth. During subsequent growth in SS medium, adenylate cyclase activity is increased more than tenfold by addition of activator. Soluble (40,000 $\times$ g supernatant) adenylate cyclase accumulates in the culture medium during exponential growth, but accumulation terminates when whole cell activity peaks and begins to decrease. The changes in whole cell activity require growth of the organism and do not occur when cells are incubated in saline over the same period.

It is well recognized that B. pertussis undergoes phenotypic degradation. With this background, we have assayed the adenylate-cyclase activity of 1) fully active, laboratory-adapted strains; 2) fresh clinical isolates; 3) serially passed clinical isolates; and 4) fully degraded phase IV strains. When cultures were grown on BG agar, one serially passaged and all degraded strains were found to have adenylate cyclase activity reduced by 10^2 to 10^4-fold. In addition, when these strains were transferred to SS medium, the reduced level of adenylate cyclase was unaffected by addition of activator in vitro. It is not possible to determine at present whether the defect associated with degradation involves loss of enzymes or simply loss of responsiveness to activator.

INTRODUCTION

Based on suggestions that Bordetella pertussis organisms had adverse effects on the adrenergic function of host animals (1), the adenylate cyclase of the bacteria was studied to determine if it might be involved (2). It was discovered that the adenylate cyclase is unique among microbial and mammalian cyclases in that it is predominantly extracytoplasmic, accumulates in the culture medium during growth, and is unaffected by previously recognized adenylate cyclate regulatory agents (3,4). It has recently been shown that the enzyme is activated in vivo or in vitro by a protein molecule present in erythrocytes and several other tissues (5). Although this activator is able to increase the adenylate cyclase activity as much as 1,000-fold, its functional significance to the organism remains unknown.

Using this activator we have evaluated the time course of basal and activated adenylate cyclase activity with growth. Also, in light of the phenotypic changes known to accompany serial passage of B. pertussis in vitro (6), we have measured the adenylate cyclase activity of a number of strains in various stages of degradation. One of the serially passed fresh isolates and all of the fully degraded phase IV organisms had markedly reduced activity.

METHODS

Time course studies were done on B. pertussis BB–114, which is maintained in a lyophilized seed lot system. Organisms were cultured on modified BG agar slants containing 20% rabbit blood or modified Stainer-Scholte synthetic medium as described previously (4,5). BG-grown cells were removed from agar with a wire loop, resuspended in SS medium or saline (50ml in a 250 ml Ehrlenmeyer flask), and incubated in a shaking water bath at 35.5° C. Aliquots were removed at the times indicated, absorbance at 650 nm was measured, and the cells were separated from medium by centrifugation (40,000 $\times$ g $\times$ 30 min). Cells were resuspended in saline and assayed for adenylate cyclase activity.

Adenylate cyclase activity was assayed as described previously (3) except that the ATP regenerating system of creatine phosphate and creatine phosphokinase was omitted.[1] Intact organisms and supernatant medium were assayed with and without activator added 10 minutes before initiation of the reaction with substrate. Activator was prepared from rabbit erythrocyte lysate by Sephadex G–100 chromatography to remove the hemoglobin.

Fresh clinical isolates and their serially passed derivates were kindly provided by Dr. Charlotte Parker. Characterization of the biological and physiological properties of these strains is discussed elsewhere in these proceedings. The phase IV variants were generously provided by Dr. Jean Dolby. These strains have been subcultured and further characterized by Drs. Wesley Kloos and Walter Dobrogosz. Each strain was cultured on BG agar and/or, when possible, in SS medium. Cells were transferred to fresh medium or removed for adenylate cyclase assay daily. Organisms grown in SS medium were assayed with and without activator. Adenylate cyclase activity is expressed in nanomoles cAMP formed/min/mg protein or picomoles cAMP formed/min/10μl culture medium. Proteins were determined by the method of Lowry (7).

RESULTS

When *B. pertussis* 114 grown on a BG slant was transferred to SS medium, the culture reached stationary phase in 20–24 hours (Fig. 1). The basal adenylate cyclase activity of the organisms prior to inoculation into SS medium was 20 nmol cAMP/min/mg protein and there was no significant increase with the inclusion of activator in the assay (Fig. 2). On transfer to SS medium, however, the activity immediately decreased to 0.35 nmol cAMP/min/mg protein and continued to be unaffected by activator. During the first hour of incubation the basal activity decreased further, but the enzyme responded 100-fold to the addition of activator. After 1 hour, there was a rapid increase in both basal and activated whole cell adenylate cyclase activity; this reached a maximum at 8 hours, then decreased. At the peak of activity, activator was able to increase basal enzyme activity 30-fold.

During the 8 hours in which the whole cell adenylate cyclase activity was increasing, there was
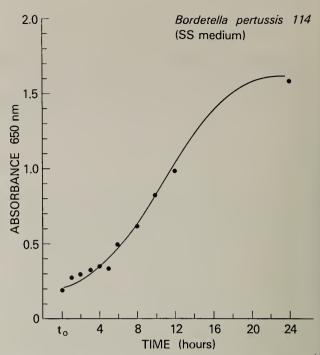
Figure 1. Time course of growth of *B. pertussis* 114 in SS medium. Exponentially growing organisms were removed from BG agar slants with a wire loop to minimize contamination with blood and inoculated into SS medium. The culture medium of 50 ml was incubated in a 250 ml flask on a shaking water bath at 35.5°C. Aliquots were removed at the times indicated for measurement of absorbance at 650 nm and for adenylate cyclase assay.

a parallel accumulation of enzyme in the culture medium (Fig. 3). After the whole cell activity peaked and began to decrease, however, there was no further accumulation of culture medium enzyme. The peak of whole cell activity and the termination of soluble enzyme accumulation appeared to coincide with the inflection point of the growth curve, representing the beginning of the end of exponential growth. When organisms from BG slants were resuspended in saline rather than SS medium and incubated under identical conditions for 24 hours, there was no growth (data not shown) and the above-described changes in adenylate cyclase activity did not occur (Fig. 4). This observation supports the suggestion that adenylate cyclase activity may be linked to the regulation of *B. pertussis* growth.

To evaluate the functional importance of this enzyme to the bacteria, we decided to search for an adenylate cyclase deficient mutant. However, screening for such an organism was difficult because the physiologic consequence of the deficiency is unknown. We chose to examine a group of organisms most likely to demonstrate a loss of adenylate cyclase, namely serially passed and degraded strains.

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[1] In recent studies it has been shown that creatine phosphokinase has a low level of stimulatory activity for *B. pertussis* adenylate cyclase and falsely elevates basal activity (Hewlett et al., article in preparation).
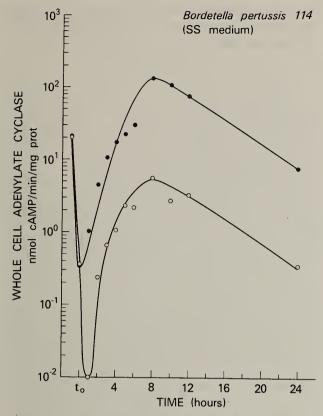
Figure 2. Time course of basal and activated *B. pertussis* 114 whole cell adenylate cyclase activity with growth. Strain 114 organisms described in Figure 1 were used. The adenylate cyclase activity of the organisms on BG agar used for inoculum is graphed to the left of time 0. Aliquots were removed at time points indicated, separated from medium by centrifugation and resuspended in saline for adenylate cyclase assay. Symbols O—O, basal; ●—●, assayed in the presence of activator.

Figure 3. Time course of basal and activated *B. pertussis* 114 soluble adenylate cyclase in the supernatant culture medium. Organisms were grown as described. Supernatant culture medium was assayed for adenylate cyclase activity after removal of organisms by centrifugation (40,000 × g for 30 min at 4° C). Symbols: O—O, basal; ●—●, assayed in the presence of activator.
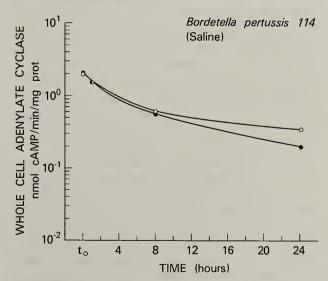
Figure 4. Time course of basal and activated *B. pertussis* 114 whole cell adenylate cyclase activity with incubation in saline. Strain 114 organisms were harvested from BG agar as described, but were inoculated into sterile saline rather than SS medium. Cells were removed and assayed as described at the time points indicated. Symbols: O—O, basal; ●—●, assayed in the presence of activator.

The above characterization of the role of growth conditions and of activator in the activity of measurable adenylate cyclase permitted a less ambiguous assessment of this enzyme in degraded strains than heretofore possible.

Four categories of *B. pertussis* organisms were cultured on BG agar and/or in SS medium (Table 1). Group I consisted of strain 114 and several other laboratory-adapted strains which maintain potency in biological activities. Group II consisted of fresh clinical isolates with few (<4) in vitro passages. Group III consisted of the same clinical isolates after multiple (>10) passages. Group IV consisted of phase IV strains from a culture collection. As expected, Group I strains were similar to 114, which has been the prototype strain in the studies of *B. pertussis* adenylate cyclase. Activity on BG medium was high, but decreased in SS medium and responded to exogenous activator with increases of

Table 1. Effect of Culture Medium and Activator on Adenylate Cyclase Activity of *B. pertussis* Strains

		Adenylate Cyclase Activity (nmol cAMP/min/mg protein)		
	Strain	BG agar	SS medium basal	+ activator
Group I [a]	BB–114	153	0.4	265
	BB–18323	59.6	2.6	101
	BB–2	N.D.	1.6	246
	BB–27	N.D.	0.5	274
Group II [b]	UT21–3	48.4	N.D. [e]	N.D.
	UT25–3	112	N.D.	N.D.
	UT26–3	59.0	N.D.	N.D.
Group III [c]	UT21–30	0.03	0.004	0.007
	UT25–14	140	0.03	106
	UT26–13	387	0.12	198
Group IV [d]	L51	0.03	0.001	0.001
	F28	0.10	0.002	0.002
		0.05	0.008	0.009

[a] Biologically active, laboratory strains
[b] Low passage clinical isolates (see Field and Parker, these proceedings)
[c] High passage clinical isolates
[d] Phase IV strains
[e] Group II organisms did not grow in SS medium.

twenty- to several hundredfold. Group II strains had high activity on BG agar, but did not grow in synthetic SS medium. Group III organisms revealed marked heterogeneity. Strain 21-30 (after 30 in vitro passages) had a markedly decreased adenylate cyclase activity when grown on BG agar. In addition, it grew well in SS medium but had similarly low basal activity that was unresponsive to activator. Strain 25-14 appeared little different from its parent strain 25-3 except that it grew well in SS medium. It appeared to be essentially similar to the Group I strains. Strain 26-13 was similar to 25-14, except that the adenylate cyclase activity on BG agar was markedly higher than all other strains tested. The significance of this difference is unknown.

Group IV strains were uniform in their decreased activity on BG medium and their lack of response to activator in SS medium. We were concerned that these organisms might represent something other than *B. pertussis* and thus have minimal measurable adenylate cyclase activity when assayed as intact organisms (4). As noted in Methods, however, these strains have been subcultured and characterized by Drs. Kloos and Dobrogosz. Strain L51 has been further characterized and shown to be *B. pertussis* by DNA hybridization by Dr. Kloos. For that reason, we chose strain L51 to carry out a complete time course of adenylate cyclase activity with growth. As

shown in Table 2 there was no increase of either basal or activated enzyme activity over the time course required for growth to stationary phase.
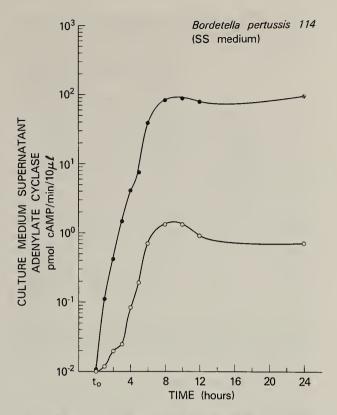
DISCUSSION

The changes occurring in the activity of *B. pertussis* adenylate cyclase with different culture media and with growth are striking, but complex. The high activity associated with growth on BG agar is now better understood, in light of the knowledge of a protein activator of the enzyme present in erythrocytes (5). Because of the rabbit blood present in BG agar, the organisms are continuously exposed to a more than adequate supply of activator. The reason for the rapid fall in activity associated with inoculation into SS medium is not known. In addition to the absence of activator, an inhibitor seems to be produced, since freshly added activator could not restore full cyclase activity at this point. In separate experiments, the early decrease in activity was shown to be unaffected by the presence of albumin in the medium.[2] The basal activity and that achieved with the addition of activator appeared to increase in parallel during growth. The coincidence of the peak of basal and activated activities of the whole cell and the end of accumulation of the enzyme in the supernatant medium with the inflection point of the growth curve is striking. Although the causal sequence of events is not yet clear, this suggests that

[2] The addition of albumin to Stainer-Scholte medium has been shown to decrease the toxicity of the medium and reduce the size of the inoculum required for growth (Dobrogosz, Ezzell, Kloos, and Manclark, these proceedings).

Table 2. Adenylate Cyclase Activity of Strain L51 During Growth

		Adenylate Cyclase Activity (nmol cAMP/min/mg protein)	
Medium	Time of Culture (hours)	basal	+ activator
BG	(pre-inoculation into SS)	0.04	0.02
SS	0	0.002	0.03
	2	0.005	0.005
	5	0.01	0.005
	8	0.002	0.013
	14	—	0.002
	24	0.002	0.002

Strain L51 was grown on BG agar, removed by wire loop and inoculated into SS medium as described for strain 114. Aliquots of organisms were removed at times indicated, separated from culture medium and assayed for adenylate cyclase activity with and without preincubation in the presence of activator.

the production of adenylate cyclase enzyme may be intimately linked to the control of growth in the organism. This hypothesis is supported by the absence of the characteristic changes in enzyme activity when the organisms are incubated in saline and do not grow.

Phenotypic variation of *B. pertussis* organisms in vitro has been recognized for almost 50 years (8,9). The observed changes include alterations in virulence, colony morphology, toxin production, antigenicity, and growth requirements (6,8,9). It is also possible to cause similar changes in the organism by manipulating the ionic environment during culture (10). In light of the apparent relationship between adenylate cyclase activity and growth and of the decreased nutritional requirements with strain degradation, it seemed reasonable to examine a series of degraded strains for possible alterations in adenylate cyclase activity. The discovery that one serially passed clinical isolate and all of the fully degraded phase IV strains had marked diminution of adenylate cyclase activity was not surprising. Indeed, this observation has recently been confirmed by Drs. Parton and Durham in studies presented in these proceedings and elsewhere.[3]

The relationship of adenylate cyclase activity to the regulation of growth and phenotypic variation with serial passage can be explained in two ways. First, adenylate cyclase may have an important regulatory function which when lost results in loss of other activities such as toxin production and virulence. This hypothesis is made less plausible in light of the absence of changes in toxicity and potency observed when organisms are grown in SS medium (resulting in markedly decreased adenylate cyclase activity). Indeed, the only apparent differences between BG- and SS-grown cells with dramatically different cyclase activities involve mild changes in cell morphology and staining characteristics. Alternatively, it is possible that the adenylate cyclase is not active in a regulatory role and

that the apparent associations with growth control are merely temporal and not causal. In this case, adenylate cyclase may simply be an enzyme lost along with a number of others with degradation of the organism.[4]

The discovery of erythrocyte activator has added a new dimension to the relationship between adenylate cyclase and strain degradation. As demonstrated in Table 1, the partially and fully degraded strains with markedly reduced enzyme activity have lost the ability to respond to activator. However, because the very low values approach the limits of resolution of the assay, it is not possible to distinguish between loss of responsiveness to activator and actual loss of the adenylate cyclase molecule. Studies are currently under way to determine if immunologic techniques can be used to make this distinction.

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[4] Further discussion of the possible mechanism involved in the strain degradation with serial passage is presented elsewhere in these procedings by Field and Parker.

REFERENCES

1. Keller, K. F.; Fishel, C. W. In vivo and in vitro manifestations of adrenergic blockade in *Bordetella pertussis*-vaccinated mice. J. Bacteriol. 94:804–811; 1967.
2. Wolff, J.; Cook, G. H. Activation of thyroid membrane adenylate cyclase by purine nucleotides. J. Biol. Chem. 248:350–355; 1973.
3. Hewlett, E. L.; Wolff, J. Soluble adenylate cyclase from the culture medium of *Bordetella pertussis*. J. Bacteriol. 127:890–898; 1976.
4. Hewlett, E. L.; Urban, M. A.; Manclark, C. R.; Wolff, J. Extracytoplasmic adenylate cyclase of *Bordetella pertussis*. Proc. Natl. Acad. Sci. USA. 73:1926–1930; 1976.
5. Hewlett, E. L.; Wolff, J; Manclark, C. R. Regulation of *Bordetella pertussis* extracytoplasmic adenylate cyclase. Adv. Cyclic Nucleotide Res. 9:621–628; 1978.
6. Wardlaw, A. C.; Parton, R.; Hooker, M. J. Loss of protective antigen, histamine-sensitising factor and envelope polypeptides in cultural variants of *Bordetella pertussis*. J. Med. Microbiol. 9:89–100; 1976.
7. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent J. Biol. Chem. 193:265–275; 1951.
8. Leslie, P. H.; Gardner, A. D. The phases of *Haemophilus pertussis*. J. Hyg. (Camb). 31:423–434; 1931
9. Toomey, J. A.; Ranta, K.; Robey, L.; McClelland, J. E. The phases or types of *H. pertussis*. J. Infect Dis. 57:49–56; 1935.
10. Lacey, B. W. Antigenic modulation of *Bordetella pertussis*. J. Hyg. (Camb.) 58:57–93; 1960.

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[3] Drs. R. Parton and J. P. Durham have recently reported similar loss of adenylate cyclase activity in phase IV strains and in organisms grown in an altered ionic environment (FEMS Microbiology Letters, in press, 1978).

Physiology of *Bordetella pertussis*

W. J. Dobrogosz, J. W. Ezzell, W. E. Kloos, and C. R. Manclark

ABSTRACT

A number of biochemical properties and growth characteristics of *Bordetella pertussis* have been examined to determine how fresh isolates (phase I strains) differ from degraded (phase IV strains) and derive synthetic liquid and agar media capable of supporting growth of both types of strains starting from low inocula. *B. parapertussis* and *B. bronchiseptica* strains were also used to obtain a genus-wide perspective on these matters.

Among the various pertussis strains tested no differences were detected between phase I and phase IV variants with regard to their content of superoxide dismutase, catalase, and peroxidase, or their ability to oxidize glutamate, their primary growth substrate in Stainer-Scholte medium. They did not vary in fatty acid composition. They also appeared to have identical plasmid components and their DNA was found to be homologous. Other components and functions, however, differed significantly. At least four outer membrane proteins plus cytochrome d–629 were found in phase I but not phase IV strains. Phase IV strains were more resistant than phase I strains to several antibiotics. Phase IV strains could also grow on a modified Stainer-Scholte agar medium (containing 1% enzymatically hydrolyzed casein), but phase I strains could not grow on it unless it was detoxified by inclusion of bovine serum albumin or charcoal. Phase I strains produced cyclic AMP, whereas the few phase IV strains examined in this regard seemed unable to do so.

Outer membrane components of *B. parapertussis* and smooth strains of *B. bronchiseptica* resembled those of phase I *B. pertussis* strains in many respects. However, rough strains of *B. bronchiseptica* and C-mode *B. parapertussis* strains resembled phase IV *B. pertussis* strains.

INTRODUCTION

Virulent strains of *Bordetella pertussis,* often referred to as phase I strains, are known to contain several biologically active components that interact in some way with the host during pathogenesis (1). These components, listed in Table 1, include a virulence factor(s), histamine-sensitizing factor (HSF), lymphocyte-promoting factor (LPF), hemagglutinating factor, protective antigen, hemolytic factor, mitogenic factor, and a dermonecrotic toxin. Through some still undetermined mechanism, phase I strains can undergo a shift or degradation to an avirulent form referred to as phase IV (2). This shift is generally associated with loss of the factors listed in Table 1 (3).

The research described in this report summarizes our efforts to determine the physiological and biochemical changes that accompany the transition from a phase I to a phase IV status. Identification of such changes should aid in the elucidation of the mechanism(s) responsible for this shift. Understanding this mechanism would then hopefully enable one to better cope with or stabilize the degradation process so that more reliable and perhaps safer pertussis vaccines can be developed.

Several differences between phase I and phase IV strains have already been noted, including differences in growth on solid and liquid media (3,4); differences in envelope polypeptides (5); and differences in adenylate cyclase synthesis (Hewlett and Manclark, personal communication). We will elaborate further on some of these areas and will describe some additional differences as well.

Table 1. Biologically Active Components and Properties of *B. pertussis*

Component/Property	Phase I	Phase IV
Virulence	+	−
Histamine sensitizing factor (HSF)	+	−
Lymphocyte promoting factor (LPF)	+	−
Hemagglutinating factor (HF)	+	−
Protective antigen (PA)	+	−
Hemolysis	+	−
Mitogenic factor	+	−
Dermonecrotic toxin	+	−

+, property detected; −, property not detected

RESULTS AND DISCUSSION

Table 2 lists some of the properties and functions of phase I and phase IV strains we have examined so far that can be classified as being either altered or unaltered by phase transition. Both phase types have essentially identical genetic homology, plasmid components, and fatty acids. Their ability to handle oxygen toxicity through the enzymes superoxide dismutase, catalase, and peroxidase seems unaltered by the transition. On the other hand, certain outer membrane proteins and cytochrome d-629 are not produced, or are produced at much lower concentrations, in phase IV strains in comparison to phase I strains. The former are less sensitive to certain antibiotics, grow in nondetoxified synthetic media and appear unable to synthesize cyclic AMP. These differences are described in more detail below.

The various strains of *B. pertussis* used for these comparative analyses are listed in Table 3. With the exception of strain F–28 (phase IV), which was obtained from K. Muse (North Carolina State University, Raleigh, N. C.), all strains were obtained from C. R. Manclark (Bureau of Biologics, FDA, Bethesda, Maryland). The phase designations shown are those which were reported to us except for strains C 188-7 and CA 226-3, which were designated in our laboratory as being in an intermediate or unclassified phase.

Table 2. Characteristics of Phase I and IV *B. pertussis*

Not Different	Different
Superoxide dismutase	Outer membrane proteins
Catalase	Cytochrome d–629
Peroxidase	Antibiotic sensitivity
Plasmids	Growth
DNA homology	Cyclic AMP
Fatty acids	

Table 3. *B. pertussis* Strains and Phase Designation

Strain	Phase Designation
BB–114	I
BB–183	I
BB–143	I
BB–134	I
BB–174	I
18530	I
BB–27	I
BB–181	I
C188–7	unclassified
CA226–3	unclassified
L–51	IV
NCTC 10902	IV
F–28	IV

Determination of Superoxide Dismutase, Catalase, and Peroxidase

It is now well established that oxygen can be toxic in living systems, and although this toxicity is particularly evident in obligate anaerobes, it applies to all life forms (6). Aerobic organisms such as *Bordetella* species have adaptive mechanisms to cope with this toxicity, which is caused by the intermediate reduction forms of oxygen, namely, the superoxide anion radical ($O_2 \cdot$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH \cdot$). Two types of adaptive mechanisms have been developed by organisms to defend themselves against these agents. The first avoids the generation of these intermediates by use of cytochrome-cytochrome oxidase systems, which insure a four-electron reduction of O_2 with no formation of the toxic intermediates. The second mechanism involves production of enzymes that detoxify these intermediates. The superoxide radical is eliminated by a metaloenzyme called superoxide dismutase (SOD), and hydrogen peroxide is eliminated by the enzymes catalase and peroxidase.

It was hypothesized that some of the difficulties encountered in growing *B. pertussis* strains and the problem of strain degradation to phase IV-type variants could perhaps be related to the inability of phase I or fresh isolates to cope with these toxic agents. In this connection a collaborative study was undertaken with the laboratory of Dr. Irwin Fridovich (Duke University, Durham, North Carolina) to determine levels of SOD, catalase and peroxidase in phase I and IV strains. Strains representative of both phases were grown to early stationary phase in modified Stainer-Scholte medium (MSS). Two strains (BB–114 and L–51) were grown with and without 5 mM cyclic AMP included in the medium. The levels of the three enzymes detected are shown in Table 4.

Table 4. Superoxide Dismutase, Catalase and Peroxidase Levels in *B. pertussis*

Strain Designation	Phase	cAMP 5mM	SOD	catalase	peroxidase
BB–114	I	–	35.2	20.0	0
		+	36.2	23.0	0
BB–182	I	–	37.3	0	0
BB–143	I	–	36.2	0	0
BB–134	I	–	46.9	0	0
L–51	IV	–	51.6	6.0	0
		+	27.8	2.0	0
NCTC–10902	IV	–	36.4	5.0	0

It can be seen that all strains tested had high levels of SOD (*E. coli* K12 has 12–15 units/mg cell protein) but were devoid of detectable peroxidase activity. The catalase content was variable, and in at least one strain (strain L–51), it appeared that cyclic AMP may have repressed both SOD and catalase synthesis. We tentatively conclude from these data that degradation of strains from phase I to phase IV is not associated with changes in these three enzymes.

Plasmids and and DNA Homology

As reported by Kloos et al. at this symposium, a large number of *B. pertussis* strains obtained from the Bureau of Biologics culture collection and others derived from fresh clinical sources were screened for the presence of plasmid DNA using agarose gel electrophoresis techniques (7). Most of the *B. pertussis* strains tested contained a small cryptic plasmid of approximately 3 megadaltons molecular mass. At this time, no correlation between the presence or absence of plasmid with the phase or virulence of *B. pertussis* strains has been detected.

Using the hydroxyapatite batch technique of Brenner and coworkers (CDC-Atlanta, Georgia) we also found that the DNAs of both phases are completely homologous. It is apparent from these studies that the degradation of phase I strains to phase IV is not due to loss of chromosomal or extrachromosomal genetic material.

Fatty Acid Analysis

Fatty acids of bacteria and other organisms are known to be subject to quantitative and qualitative changes due to media composition, temperature, strain differences, etc. Since phase I and phase IV strains differ in media requirements, virulence, etc., we felt that their fatty acid composition might in some way reflect this difference. Fatty acid profiles

were determined using the procedure of Dunlap and Perry (8). The results of these investigations are shown in Table 5. These data indicate no significant or consistent differences between phase I and phase IV strains with respect to their total fatty acid composition. Their fatty acid composition was also unaffected by growth in the presence of 2.5 mM cyclic AMP (data not shown).

Outer Membrane Proteins

It is well established that phase IV strains are more resistant than phase I strains to toxic substances in laboratory media, to fatty acids, and to bacteriocins (4,9). The greater resistance of phase IV strains to penicillin, ampicillin, streptomycin, erythromycin, and tetracycline and to Tween 80 has been shown in this laboratory and will be discussed later. In recent years several investigators have reported that certain outer membrane (OM) proteins in *Escherichia* and *Salmonella* species form pores through the OM and that mutant strains lacking these proteins have greater resistance to a variety of toxic substances such as copper sulfide (10) and antibiotics such as cephaloridine (11).

We analyzed the OM proteins of *B. pertussis* strains representing both phase types. The proteins were isolated, solubilized, and electrophoretically separated, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) as described by Schnaitman (12). The results of these analyses are shown in Figure 1. Graphs A, B, and C are gel scans of the OM proteins of the phase I strains BB–114, 18530, and BB–174. Several other phase I strains have also been analyzed with essentially identical results (data not shown). The OM proteins of all phase I strains were found to possess at least 10 readily detectable proteins or protein subunits. Four of these are either not produced or produced

Table 5. Fatty Acid Composition of *B. pertussis*

Strain and Phase	Fatty Acid Carbon Length/Relative Retention Time (sec)									
	C14 0.53	IU a 0.72	C16 1.0	C16:1 1.2	IU 1.75	C18 1.9	C18:1 2.2	IU 2.7	C20 3.7	IU 4.3
BB–114 (I)	3.1 b	0.3	40.3	38.2	—	11.7	5.8	0.5	0.4	—
BB–182 (I)	3.6	0.3	44.4	42.7	0.9	8.0	1.3	—	—	—
BB–143 (I)	3.9	—	40.2	43.3	—	10.6	0.9	—	1.1	—
BB–134 (I)	4.9	0.7	39.2	35.4	3.1	7.7	3.0	3.2	—	2.7
L–51 (IV)	5.1	—	45.1	42.8	—	6.2	1.9	—	—	—
NCTC–10902 (IV)	6.7	—	39.9	39.4	—	12.2	1.2	—	1.7	—

a Identity unknown
b Percentage of total
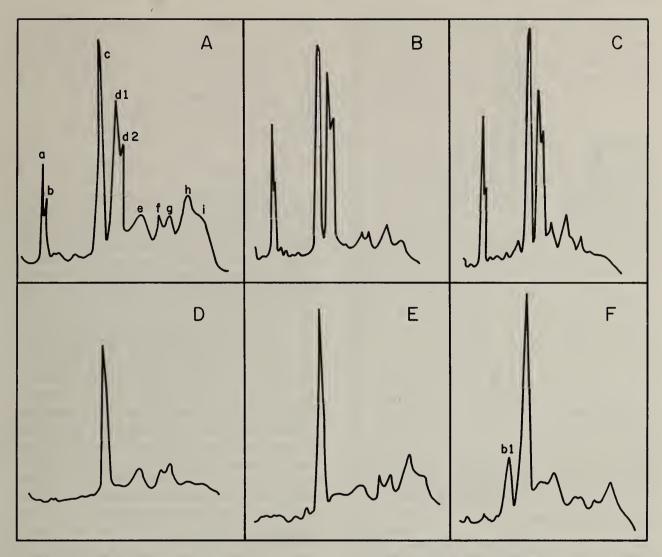
Figure 1. Gel scans of OM proteins of *B. pertussis*. Phase I strains: BB–114 (A), 18350 (B), and BB–174 (C). Phase IV strains: NCTC 10902 (D), L–51 (E), and F–28 (F). Protein profile of strain BB–114 (A) has been labeled to establish nomenclature.

in much lower concentrations in phase IV strains (L–51, NCTC 10902 and F–28) as shown in graphs D, E, and F of Figure 1. The molecular masses of these four proteins, which have been designated as proteins a, b, d1, and d2, were estimated to be 98, 88, 30, and 27.5 kilodaltons respectively. Proteins d1 and d2 may be identical with the two proteins (B and C) described by Parton and Wardlaw (5,13). These investigators reported that proteins B and C are present only in phase I cells and have molecular masses of 30 and 28 kilodaltons respectively. However, as stated above, our analyses revealed two higher molecular weight proteins (a and b) also present in phase I but not in phase IV strains. The difference in results can be attributed to the somewhat different procedures used in each laboratory.

The data presented in Figure 2 show a typical phase I OM protein profile in comparison with a sample of pertussigen supplied by J. Munoz (NIAID, Hamilton, Montana) and a sample of LPF supplied by S. I. Morse (Downstate Medical Center, Brooklyn, N. Y.). It appears from these results that the latter two samples are relatively rich in the four OM proteins associated only with phase I strains. The slight offset of relative mobilities of these latter proteins can be explained on the basis of slight differences in sample preparations.

In data not shown here, we were able also to demonstrate that the four proteins unique to the phase I strains were severely repressed when these cells were converted to the C-mode form (14) by growth in the presence of 20 mM $MgSO_4$. As will be described later, this conversion also produces increased resistance to certain antibiotics.
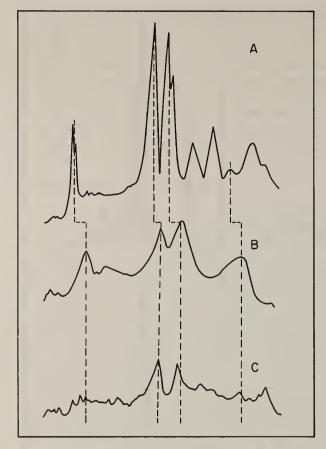
Figure 2. Gel scans of OM proteins from *B. pertussis* strain BB–114 (A), purified leukocytosis promoting factor from S. I. Morse (B), and "pertussigen" from J. Munoz (C).

Figure 3. **Difference spectra of strains BB–114 and NCTC 10902.** Shown are difference spectra of strains BB–114 and NCTC 10902 determined using a Beckman Acta V dual beam spectrophotometer. Whole cells, adjusted to 1.0 gram wet cell weight/5ml HEPES buffer (pH 7.2), were oxidized in both the sample and reference cuvettes by adding 10 μl 3% hydrogen peroxide per 2.5 ml of cell suspension to determine the oxidized minus oxidized base line (–•–•–). The reduced minus oxidized spectrum (———) was determined after addition of a few grains of dithionite to the sample cuvette. The CO-reduced minus reduced spectrum (– – – –) was determined after reducing the reference cuvette with dithionite and bubbling the previously reduced sample cuvette with carbon monoxide for 5 min. Depicted in the insert is an expanded portion of the spectrum.

Cytochrome Content

During our investigations we noted that cell pellets derived from centrifuged phase IV cells had a more pronounced reddish cast than those from phase I cultures. This and other findings suggested that differences in their cytochrome components might exist. The data presented in Figure 3 show the difference spectra for a typical phase I strain (BB–114) and a typical phase IV strain (NCTC–10902). All strains examined possessed cytochromes A–603, b–560, c–553, and cytochrome o. Only phase I strains, however, possessed cytochrome d–629 as well. As was found with the OM proteins unique to phase I cells, the d–629 component was repressed in these cells when they were converted to the C-mode by growth in the presence of 20 mM MgSO$_4$ (data not shown). Cyclic AMP additions to growth media had no effect on cytochrome synthesis whether added with or without 20 mM MgSO$_4$.

Antibiotic Sensitivity

As mentioned earlier, phase I and IV strains differed in their sensitivity to the antibiotics penicillin, ampicillin, streptomycin, erythromycin, and tetracycline. This sensitivity was determined by measuring the zones of growth inhibition circumscribing discs impregnated with these antibiotics and placed on the surface of Bordet-Gengou medium supplemented with 15% defibrinated sheep blood. As shown in Table 6, phase IV strains could

Table 6. **Effect of Antibiotics and Antibiotics Plus Mg++ on Growth of *Bordetella pertussis***

Strain	Zone of Inhibition [a]									
	Pen [b]	Pen + Mg	Amp	Amp + Mg	Strep	Strep + Mg	Eryth	Eryth + Mg	Tet	Tet + Mg
B. pertussis BB–114	4.5	12.5	6.5	13.0	8.5	5.0	20.5	12.5	23.0	15.5
BB–182	3.5	7.5	13.5	16.5	4.5	3.5	22.5	11.0	18.5	13.5
NCTC 10902	0	0	0	0	1.0	0	1.0	0	8.5	0
L–51	0	0	0	0	2.5	0	1.0	0	5.0	0

[a] Zone of inhibition in mm, measured from edge of disc to edge of inhibition zone.
[b] Antibiotic concentrations: penicillin, 2 units; ampicillin, 2μg; streptomycin, 10 μg; erythromycin, 2 μg; tetracycline, 5 μg.

be differentiated from phase I strains by their greater resistance to these antibiotics. Furthermore, we were able to show that MgSO$_4$ additions altered these sensitivity patterns in such a manner that the phase I cells tested became more sensitive to penicillin and ampicillin, whereas both phases became more resistant to streptomycin, erythromycin, and tetracycline. These results suggest that such tests could prove useful as a rapid means for distinguishing between the two phases.

The role of MgSO$_4$ in enhancing sensitivity to penicillin and ampicillin and decreasing sensitivity to the other antibiotics is unknown. It can be noted that increased resistance of *E. coli* and *Staphylococcus aureus* to tetracycline in the presence of Mg^{++} has been reported by Sompolensky and Samra (15). It was speculated that this cation interfered with tetracycline uptake.

Cyclic AMP Determinations

Although our analyses of cyclic AMP production by these strains are still incomplete, we can report that phase I strains BB–114, BB–182 and BB–181 show cyclic AMP production. The phase IV strain L–51 appears to be unable to synthesize detectable levels of this cyclic nucleotide.

Analyses of Unclassified Strains

The two strains designated as C188–7 and CA226–3 are unclassified with respect to their phase status. They may, however, represent an intermediate state in the degradation of the phase I strain BB–27 in that they were found to possess a mixture of the characteristics described above.

All phase I strains used in this study were unable to grow on a modified Stainer-Scholte agar medium supplemented with 1% enzymatically hydrolyzed casein. When this medium was detoxified by inclusion of either bovine serum albumin or activated charcoal, phase I cells grew well. Phase IV cells, however, grew well with or without inclusion of these detoxifying agents. We are presently using growth on this medium as an additional criterion for distinguishing between phase I and phase IV cultures. The latter cultures will grow with or without the presence of detoxifying agents and the former will not. In this respect the unclassified strains resembled phase IV strains. They produced cyclic AMP, showed hemolysis on Bordet-Gengou medium, and exhibited an antibiotic sensitivity pattern characteristic of phase I strains. Their content of the high molecular weight OM proteins (a and b) also resembled phase I cells. However, their content of OM proteins d1 and d2 cytochrome d–629 appeared to be intermediate between phase I and phase IV strains. This was indicated by the low but detectable levels of these proteins in these particular strains.

Studies on *B. parapertussis* and *B. bronchiseptica*

Essentially all of the analyses conducted on the *B. pertussis* strains listed in Table 2 were applied to a study of the *B. parapertussis* and *B. bronchiseptica* strains listed in Table 7. *B. parapertussis* strains uniformly showed protein profiles closely resembling those of phase 1 *B. pertussis* strains except that two additional high molecular weight proteins (112 and 81 kilodaltons) were detected. The smooth strains of *B. bronchiseptica* also had similar profiles to *B. pertussis* except that the two high molecular weight proteins (a and b) found in *B. pertussis* were not present. The rough strains of *B. bronchiseptica* resembled phase IV *B. pertussis* in that proteins d1 and d2 were missing.

Although "degraded" strains of *B. parapertussis* were not available, we found that C-mode cells of this species had attenuated levels of proteins d1 and d2 as well as those with molecular masses of 112, 95, 88, and 81 kilodaltons. The d1 and d2 proteins were also attentuated in C-mode *B. bronchiseptica*. These data suggest that the control mechanisms exerted over these OM proteins are similar in all three species.

Difference spectra of *B. parapertussis* were similar to those of phase I pertussis strains, suggesting that both species have an identical cytochrome system. However, difference spectra of *B. bronchiseptica* more closely resembled those of phase IV pertussis strains in that we have been unable to detect a cytochrome d-629 peak.

Table 7. *Bordetella parapertussis* and *Bordetella bronchiseptica* Strains Tested

Species	Strain
B. parapertussis	A5555 (CDC, Atlanta, Ga.)
	21499 (C. R. Manclark, Bureau of Biologics, FDA)
	NK–CDC (N. King, N. C. Dept of Public Health, Raleigh, N. C.)
B. bronchiseptica	A (smooth)
	B (smooth)
	C (rough) (R. A. Goodnow, Burns-
	D (rough) Biotec, Omaha, Neb.)
	E (smooth)
	F (smooth)

SUMMARY

Considerable debate exists concerning the nature of the degradation process through which freshly isolated strains undergo shifts to less virulent or avirulent forms. As for the nomenclature describing these virulent and avirulent forms, some investigators (16) contend that smooth and rough designations are sufficient while others (2,4) hold that multiple intermediate states exist and must be so designated. Our studies reported here tend to favor the latter view since some strains tested were clearly not classifiable as either phase I or phase IV.

The primary purpose of this study, however, was to establish additional criteria by which virulent phase I-type strains can be distinguished from the totally degraded phase IV-type strains. Obviously, such criteria must be available before any serious attempt is made to understand the mechanism(s) that underlies the degradation process in these organisms. Through our efforts presented here and those reported by others, a growing list of such criteria or "markers" of strain degradation is now available.

Not surprisingly, the OM proteins provide excellent markers, with at least four of them (a, b, d1 and d2) present in phase I but not in phase IV strains. That these proteins may be one or more of the biologically active components listed in Table I is indicated by their apparent presence in pertussigen and LPF preparations. The presence of cytochrome d–629 in phase I but not in phase IV strains indicates a coordinate shift with regard to certain respiratory proteins. Of course, we cannot at this time exclude the possibility that d–629 component is one of the four OM proteins unique to phase I cells. All these proteins are present at reduced levels in the unclassified strains tested and are severely repressed in C-mode cells.

As for the mechanism(s) involved in phase transition in this organism, it would appear that plasmid functions are not involved. The role of cyclic AMP, on the other hand, is less clear. So far we have been unable to alter any biochemical or physiological property of either phase I or phase IV cultures by addition of up to 10 mM cyclic AMP to the culture medium. It is possible that these cells are unable to transport exogenous cyclic AMP across the cytoplasmic membrane, thus accounting for its lack of efficacy in this regard. All phase I but not phase IV strains examined to date possess adenylate cyclase activity (Hewlett et al., these proceedings) and produce cyclic AMP. The fact that this cyclic nucleotide is known to play a key role in genetic transcription in other Gram-negative bacteria, compels us to keep open the possibility that cyclic AMP regulation is involved in the *Bordetella* strain degradation process and the X-mode/C-mode reversible transition as well. Obviously, further investigation will be needed to determine its role, if any, in these phenomena.

The findings in this report support the premise that certain OM proteins in *B. pertussis* may form pores through which toxic substances in laboratory media enter the cell. As stated above, phase IV strains were missing at least four OM proteins normally found in phase I strains and were also resistant to toxic substances in laboratory media and to antibiotics. If this greater resistance is due to one or more OM proteins forming pores in *Bordetella,* as has been shown in other Gram-negative organisms, then selective pressure for pore deficient mutants by toxins in laboratory media may serve as the basis for degradation of phase I or fresh isolates to phase IV or degraded strains.

REFERENCES

1. Morse, S. I. Biologically active components and properties of *Bordetella pertussis*. Adv. Appl. Microbiol. 20:9–26; 1976.
2. Leslie, P. H.; Gardner, A. D. The phases of *Haemophilus pertussis*. J. Hyg. (Camb.) 31:423–434; 1931.
3. Aprile, M. A. A reexamination of phase IV *Bordetella pertussis*. Can. J. Microbiol., 18:1793–1801; 1972.
4. Parker, C. Role of the genetics and physiology of *Bordetella pertussis* in the production of vaccine and the study of host-parasite relationships in pertussis. Adv. Appl. Microbiol. 20:27–42; 1976.
5. Parton, R.; Wardlaw, A. C. Cell envelope proteins of *Bordetella pertussis*. J. Med. Microbiol. 8:47–57; 1975.
6. Fridovich, I. Superoxide dismutases. Ann. Rev. Biochem. 44:147–159; 1975.
7. Currier, T. C.; Nester, E. W. Isolation of covalently closed circular DNA of high molecular weight from bacteria. Anal. Biochem. 76:431–444; 1976.
8. Dunlap, K.; Perry, J. J. Effect of substrate on the fatty acid composition of hydrocarbon utilizing microorganisms. J. Bacteriol 94:1919–1923; 1967.
9. Litkenhous, C.; Liu, P. V. Bacteriocin produced by *Bordetella pertussis*. J. Bacteriol. 93:1484–1488; 1967.
10. Lutkenhaus, J. F. Role of a major outer membrane protein in *Escherichia coli*. J. Bacteriol. 131:631–637; 1977.
11. Nikaido, H.; Song, S. A.; Shaltiel, L.; Nurminen, M. Outer membrane of Salmonella. XIV. Reduced trans-

membrane diffusion rates in porin-deficient mutants. Biochem. Biophys. Res. Commun. 76:324–330; 1977.

12. Schnaitman, C. Outer membrane proteins of *Escherichia coli*. I. Effects of preparative conditions on the migration of protein on polyacrylamide gels. Arch. Biochem. Biophys. 157:541–552; 1973.

13. Wardlaw, A. C.; Parton, R.; Hooker, M. J. Loss of protective antigen, histamine sensitizing factor and envelope polypeptides in cultural variants of *Bordetella pertussis*. J. Med. Microbiol. 9:89–100; 1976.

14. Lacey, B. W. Antigenic modulation of *Bordetella pertussis*. J. Hyg. (Camb.) 58:57; 1960.

15. Sompolinsky, D.; Samra, Z. Influence of magnesium and manganese on some biological and physiological properties of tetracycline. J. Bacteriol. 110:468–476; 1972.

16. Standfast, A. F. B. The phase I of *Haemophilus pertussis*. J. Gen. Microbiol. 5:531–545; 1951.

Changes in Envelope Proteins and Correlation with Biological Activities in *B. pertussis*

A. C. Wardlaw and R. Parton

ABSTRACT

Isolated cell envelopes from several strains of phase I *B. pertussis* of various serotypes showed almost identical patterns of polypeptide bands when subjected to SDS-polyacrylamide gel electrophoresis. Envelopes from phase IV strains were found to be deficient in two major components of molecular weights 28,000 and 30,000. These bands were also absent from envelopes of C-mode cells, prepared by growing phase I strains in a high-magnesium, low-sodium medium, and cells grown in a high-nicotinic acid medium. All variants examined also lacked protective antigen, histamine-sensitizing factor, lymphocytosis-promoting factor, heat-labile toxin, adjuvant for reaginic antibody production and for induction of hyperacute autoallergic encephalomyelitis, and adenylate cyclase activity. Although the relationship between these activities at the molecular level has not been fully elucidated, at least four separate components of the *B. pertussis* cell appear to be lost during the variation processes. Which, if any, of the activities is represented by the 28,000 and 30,000 molecular weight envelope polypeptides has not yet been determined.

RATIONALE FOR THE INVESTIGATION

In recent years, much progress has been made in the isolation of some of the important components of *B. pertussis* such as the histamine-sensitizing factor (HSF) (1,2), lymphocytosis-promoting factor (LPF) (3), the hemagglutinins (HA) (4) and islet-activating protein IAP (5). However, the most important component for vaccination, the protective antigen (PA), remains elusive. There may be several reasons for this: the cumbersome nature of the mouse protection test; the technical difficulties in separating membrane components; the possibility that the ratio of immunologic and pharmacologic activities in a given cell fraction may vary greatly depending, for example, on state of aggregation or solubility; the possibility that PA may not be a single component and that full protective activity may require the joint effect of two or more distinct entities.

In any event, it would clearly be advantageous to have a rapid, presumptive test for PA or other activities to assist fractionation studies. We have addressed ourselves to the possibility that electrophoretic analysis of envelope proteins of variant strains may be useful in this context. Our work has been based on the assumptions that the PA and certain other biologically active components are cell envelope proteins and that an investigation of the range of activities of variant strains may help

clarify the apparent relationships between these activities.

VARIATION IN *B. pertussis*

Our experimental approach has made use of the well known propensity of *B. pertussis* to undergo variation (6–10). At least two distinct types of variation process have been described. The first is typified by the phase variation of Leslie and Gardner (6) in which the organisms apparently undergo a series of mutational changes. The resulting "degraded" strains, the so-called phase IV variants, are avirulent and lack PA (11,12), HSF (12,13) and agglutinogen (14) characteristic of freshly isolated (phase I) strains.

The process of antigenic modulation described by Lacey (8), however, appears to involve only phenotypic changes in that it is freely reversible by alteration of cultural conditions. Conventionally grown cultures with PA, HSF, LPF, etc. are described as X-mode, whereas cultivation on a high-magnesium, low-sodium medium (C-medium) yields C-mode cells, which lack these properties (15–17). A similar reversible variation may also be induced in a high-nicotinic acid medium (17,18).

The question arises: do these variants show regular and reproducible changes in their cell envelope proteins that are paralleled by changes in their biological activities?

RESULTS

Envelope Protein Profiles

The envelope protein profiles of a large number of phase I strains and their variants have been obtained by polyacrylamide gel electrophoresis in the presence of SDS. Two distinct patterns have emerged (16,17). After solubilization of envelopes in SDS and mercaptoethanol at 100°C for 5 minutes, phase I or X-mode cultures typically have prominent polypeptide bands with apparent molecular weights of 28,000 and 30,000; phase IV, C-mode, and high-nicotinic acid cultures consistently lack these bands or show much diminished amounts (Fig. 1). Other minor, less consistent differences between strains have been observed, but except for the 28,000 and 30,000 molecular weight bands the phase I, phase IV, and C-mode profiles are essentially identical. No
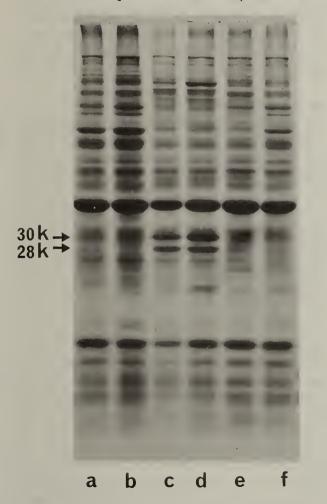
significant differences in protein profiles could be attributed to known difference in serotypes of the strains used. The two types of profiles can also clearly be seen with use of solubilized whole cells, without the need for separation and purification of envelopes.

An unusual property of the phase I specific polypeptide bands is their behavior with different solubilization temperatures. After solubilization of envelopes in SDS at 37°C for 30 minutes, the bands migrated faster with lower apparent molecular weights. Similar "heat-modifiable" proteins have been demonstrated in the outer membranes of other Gram-negative bacteria. This property may reflect some unusual structural configuration (19) and possibly a specialized functional role in the envelope.

PA and HSF Activities

All phase I cells had 28,000 and 30,000 molecular weight envelope polypeptides and PA and HSF in 56°C-heated suspensions. In contrast, when the same bacterial strains were cultured in Hornibrook-type medium modified to give C-mode cultures, the PA and HSF disappeared in parallel with the bands (Table 1). Similar parallelism was observed when media containing different amounts of magnesium and sodium ions were used, when the concentration of nicotinic acid was increased (17), or when cultures were transferred back and forth between X- and C-media with sampling after different periods of incubation (Idigbe, Parton, and Wardlaw, unpublished). It appeared that a time during which there were four cell divisions in C-medium was required to effect complete conversion from X-mode to C-mode.

Lymphocytosis-Promoting Factor

LPF is known to be absent from phase IV strains. When strain 18334 was grown in C-medium, LPF activity also disappeared in parallel with PA, HSF,

Figure 1. Envelope protein profile of *B. pertussis* strain **GL353Z**: (a) Phase I grown in high-nicotinic acid medium; (b) phase IV grown in X-medium; (c) phase I grown in X-medium; and strain 134 grown in (d) X-medium; (e) C-medium; (f) high-nicotinic acid medium.

Table 1. Protective Antigen (PA) and Histamine-Sensitizing Factor (HSF) in X-Mode and C-Mode *B. pertussis* Strains (17)

Strain Number	Potency (%) of C-Vaccine Relative to X-Vaccine	
	PA	HSF
18334	4.1	3.0
134	2.6	9.0
L-84	8.2	4.3
10739	16	18
GL353Z	<1.6	18

and the 28,000 and 30,000 molecular weight gel bands (Idigbe, unpublished observations).

Heat-Labile Adjuvant (HLAdj) for Reaginic Antibody Production and Hyperacute EAE

C-mode cell suspensions of strain 18334 heated at 56° C for 30 minutes were found to be much less adjuvant active than the X-mode in two systems (Wardlaw, Parton, Bergman, and Munoz, submitted for publication): stimulation of reaginic antibodies to ovalbumin in HAM/1CR mice (Fig. 2) and induction of hyperacute experimental autoallergic encephalomyelitis (EAE) to guinea pig spinal cord in Lewis rats (Fig. 3).

Heat-Labile Toxin

Whereas PA, HSF, and LPF are generally considered to be envelope components, the heat (56° C)-labile toxin (HLT) appears to be cytoplasmic (20) (although a periplasmic location cannot be excluded). HLT is not therefore considered part of the macromolecule(s) assumed to have PA, HSF, LPF, HA, and IAP activities. In considering the mechanism of antigenic modulation, account has to be taken of our observations (21) that HLT is almost completely absent from C-mode cells (Table 2).
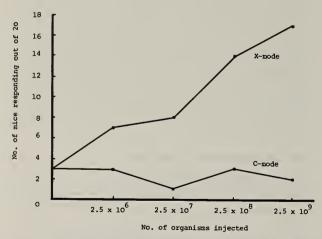
Figure 2. **Comparative adjuvant activity of X-mode and C-mode** *B. pertussis* **vaccines for induction of IgE antibody.** Vaccines were standardized, before heating at 56° C for 30 minutes, by comparison with the International Opacity Standard. One International Opacity Unit is assumed equivalent to 10⁹ organisms per ml. Groups of 20 HAM/1CR mice were injected intraperitoneally with 200 μg ovalbumin mixed with graded doses of *B. pertussis* strain 18334 vaccines. After 21 days, mice were bled and the individual sera tested for presence of IgE antibodies by the passive cutaneous anaphylaxis (PCA) test in hairless mice. A 48h sensitization period was used. (Wardlaw, Parton, Bergman, and Munoz, submitted for publication).
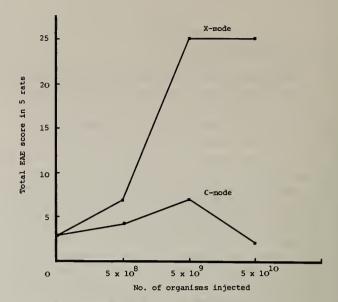
Figure 3. **Comparative adjuvant activity of X-mode and C-mode** *B. pertussis* **vaccines for induction of EAE.** Groups of female Lewis rats were injected intraperitoneally with 25 mg guinea-pig spinal cord emulsion mixed with various doses of *B. pertussis* strain 18334 vaccines and observed daily for symptoms of hyperacute EAE (Wardlaw, Parton, Bergman, and Munoz, submitted for publication).

Adenylate Cyclase Activity

In view of the recent demonstration of high levels of adenylate cyclase activity in *B. pertussis* (22,23) and the known role of this enzyme in the regulation of certain cellular functions in other prokaryotes (24) it was clearly of interest to measure its activity in phase IV and C-mode cultures. In a series of strains so investigated (26) a consistent and familiar pattern emerged: Phase I cells had high levels of adenylate cyclase activity, whereas the corresponding phase IV and C-mode cells had only trace amounts (Table 3). From its reported properties (23), this enzyme is probably quite distinct from the other

Table 2. **Comparison of HLT and HSF Activities of** *B. pertussis* **Strain 18334 C-Mode Cells**

Experiment Number	Potency (%) of C-Mode Preparation Relative to the X-Mode Preparation	
	Toxicity	HSF
1	3.7	0.7
2	9.3	0.5
3	4.0	1.9
4	3.7	0.4
5	4.1	<5.0

For toxicity testing, groups of 10 HAM/1CR mice were injected intraperitoneally with twofold serial dilutions of cells disrupted in an X-Press. Deaths were recorded over the next 3 days (21).

Table 3. Comparison of Adenylate Cyclase Activity in Cells of *B. pertussis* Strains and Their Variants

B. pertussis Strain	Phase	Mode	Adenylate Cyclase Activity (nmoles cyclic AMP/ mg protein/15 min)
L84	I	X	343
L84	I	C	0.10
L84	IV		0.22
D30042	I	X	128
D30042	I	C	0.18
D30042	IV		1.94
GL353Z	I	X	360
GL353Z	I	C	0.08
GL353Z	IV		0.09

biologically active components of *B. pertussis* such as LPF (3,26).

SUMMARY AND CONCLUSIONS

Phase I strains of *B. pertussis* differ consistently from phase IV, C-mode, and high nicotinic acid-grown cells in having prominent envelope polypeptides of molecular weights 28,000 and 30,000 when examined by SDS–PAGE.

Although the data are not complete, i.e. we have not examined all preparations for all activities, we have not observed any exception to the generalization that cells lacking the 28,000 and 30,000 molecular weight envelope components also lack normal amounts of PA, HSF, LPF, HLT, adenylate cyclase activity and adjuvant for reaginic antibodies and for hyperacute EAE.

Taking into account the evidence available from the most recent fractionation studies, we conclude that at least four separate cellular components are lost (or lose their activity) during phase variation and antigenic modulation. These are HLT, adenylate cyclase, a mouse-protective antigen which also has hemagglutinating activity (4), and an LPF/HSF complex with a variety of other biological activities (1–4). Which, if any, of these components make up the 28,000 and 30,000 molecular weight polypeptides is unknown, though this could be determined by electrophoresis of the purified components and envelope preparations in parallel. In any event, the above observations suggest that SDS–PAGE of envelope proteins, or even whole cell proteins, could provide a fairly rapid in vitro test for one or more phase I activities.

The identical loss of several different properties of phase I cells, including virulence, during two distinct variation processes suggests that the expression of these factors depends on a common "switch."

In view of the known regulatory function of adenylate cyclase in other organisms, where its activity can be altered by environmental factors or blocked by mutation, we suspect that this enzyme may be directly involved in the expression of the important biological activities of *B. pertussis*.

REFERENCES

1. Lehrer, S. B.; Tan, E. M.; Vaughan, J. H. Extraction and partial purification of the histamine-sensitizing factor of *Bordetella pertussis* J. Immunol. 113:18–26; 1974.
2. Munoz, J. J.; Bergman, R. K.; Cole, R. L.; Ayers, J. C. Purification and activities of pertussigen, a substance from *Bordetella pertussis* Rosenberg, P., ed. Toxins: animal, plant and microbial. Oxford and New York: Pergamon Press; 1978.
3. Morse, S. I.; Morse, J. H. Isolation and properties of the leukocytosis- and lymphocytosis-promoting factor of *Bordetella pertussis*. J. Exp. Med. 143:1483–1502; 1976.
4. Arai, H.; Sato, Y. Separation and characterization of two distinct haemagglutinins contained in purified leukocytosis-promoting factor from *Bordetella pertussis*. Biochim. Biophys. Acta 444:765–782; 1976.
5. Yajima, M.; Hosoda, K.; Kanbayashi, Y.; Nakamura, T.; Nogimori, Y.; Nakase, Y.; Ui, M. Islet-activating protein (IAP) in *Bordetella pertussis* that potentiates insulin secretory responses of rats. Purification and characterization. J. Biochem. 83:295–303; 1978.
6. Leslie, P. H.; Gardner, A. D. The phases of *Haemophilus pertussis*. J. Hyg. (Camb.) 31:423–434; 1931.
7. Standfast, A. F. B. The phase I of *Haemophilus pertussis*. J. Gen. Microbiol. 5:531–545; 1951.
8. Lacey, B. W. Antigenic modulation of *Bordetella pertussis*. J. Hyg. (Camb.) 58:57–93; 1960.
9. Cameron, J. Variation in *Bordetella pertussis*. J. Pathol. Bacteriol. 94:367–374; 1967.
10. Stanbridge, T. N.; Preston, N. W. Variation of serotype in strains of *Bordetella pertussis*. J. Hyg. (Camb.) 73:305–310; 1974.
11. Kasuga, T.; Nakase, Y.; Ukishima, K.; Takatsu, K. Studies on *Haemophilus pertussis*. Part IV. Protective potency of each phase organism of *Haemophilus pertussis* in mice. Kitasato Arch. Exp. Med. 27:49–56; 1954.
12. Aprile, M. A. A reexamination of phase IV *Bordetella pertussis*. Can. J. Microbiol. 18:1793–1801; 1972.
13. Kind, L. S. The altered reactivity of mice after immunization with *Haemophilus pertussis* vaccine. J. Immunol. 70:411–420; 1953.
14. Eldering, G.; Eveland, W. C.; Kendrick, P. L. Fluorescent antibody staining and agglutination reactions in *Bordetella pertussis* cultures. J. Bacteriol. 83:745–749; 1962.
15. Holt, L. B.; Spasojevic, V. The role of surface antigens in the protective potency of *Bordetella pertussis* suspensions as measured by the intracerebral challenge technique in mice. J. Med. Microbiol. 1:119–126; 1968.
16. Parton, R.; Wardlaw, A. C. Cell-envelope proteins of *Bordetella pertussis*. J. Med. Microbiol. 8:47–57; 1975.
17. Wardlaw, A. C.; Parton, R.; Hooker, M. J. Loss of protective antigen, histamine-sensitizing factor and envelope polypeptides in cultural variants of *Bordetella pertussis*. J. Med. Microbiol. 9:89–100; 1976.
18. Pusztai, S.; Joo, I. Influence of nicotinic acid on the antigenic structure of *Bordetella pertussis*. Ann. Immunol. Hung. 10:63–67; 1967.
19. Mizushima, S. Effect of sodium dodecylsulfate and heating on protein conformation in outer and cytoplasmic

membranes from *Escherichia coli*. Biochem. Biophys. Res. Commun. 61:1221–1226; 1974.

20. Munoz, J. J. Immunological and other biological activities of *Bordetella pertussis* antigens. Bacteriol. Rev. 27:325–340; 1963.

21. Livey, I.; Parton, R.; Wardlaw, A. C. Loss of heat-labile toxin from *Bordetella pertussis* grown in modified Hornibrook medium. FEMS Lett. 3:203–205; 1978.

22. Hewlett, E. L.; Urban, M. A.; Manclark, C. R.; Wolff, J. Extracytoplasmic adenylate cyclase of *Bordetella pertussis*. Proc. Natl. Acad. Sci. USA 73:1926–1930; 1976.

23. Hewlett, E.; Wolff, J. Soluble adenylate cyclase from the culture medium of *Bordetella pertussis*: Purification and characterization. J. Bacteriol. 127:890–898; 1976.

24. Rickenberg, H. V. Cyclic AMP in prokaryotes. Ann. Rev. Microbiol. 28:353–369; 1974.

25. Parton, R.; Durham, J. P. Loss of adenylate cyclase activity in variants of *Bordetella pertussis*. FEMS Lett. in press.

26. Morse, S. I. Lymphocytosis-promoting factor of *Bordetella pertussis:* isolation, characterization, and biological activity. J. Infect. Dis. 136:S234–S238; 1977.

Effect of Growth Conditions on the Composition and Stability of the Outer Membrane of *Bordetella pertussis*

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ABSTRACT

The composition of the outer membrane of *Bordetella pertussis* was studied in cells obtained from semi-continuous or continuous cultures in defined medium. The outer membrane, isolated in the form of vesicles by mechanical disintegration, comprised about 10% of the dry weight of the cells. It consisted of lipopolysaccharide (LPS); agglutinogens 1, 2, and/or 3; proteins; and practically all of the mouse-protective activity (MPA) of the cell. Mild acid treatment (pH 3 at 37° C) liberated protein antigens, agglutinogens, and labile deoxysugars from the LPS backbone, which comprises about 13–15% of the vesicles. The components were further separated and identified using column chromatography, crossed immunoelectrophoresis, and agglutination inhibition. Ten antigens have so far been identified, all with a molecular mass approaching 10 megadaltons; some were specific for *B. pertussis*, the others crossreacted with *Bordetella parapertussis* or *Bordetella bronchiseptica* antisera. Fractionation of the outer membrane leads to the disappearance of the MPA.

At constant medium composition, the qualitative and quantitative composition of the outer membrane was reversibly dependent upon the physiological condition of the cell. Balanced growth occurred only at 36–37° C, and only after the internal stabilization of cells during growth. Such cells had high agglutinogen content, smooth surface layers in thin sections, and relatively stable MPA during formolization and storage. Unbalanced growth occurred at lower temperatures or after transfer of the cells to a new environment and was reflected in a decrease or loss of agglutinogens, in the crumpled appearance of the surface layers in thin sections, and in the instability of MPA during formolization and storage.

INTRODUCTION

It is well known that the mouse protective activity (MPA) of *B. pertussis* culture is very variable. This variability, which concerns not only the method of vaccine production but also the standardization of the final product, is difficult to analyze since the mouse protection test (MPT) may contain serious inherent irregularities. Our aim was to study the physiology of *B. pertussis* to discover the reason for these irregularities. We have been particularly interested to know how the cultural conditions may influence the composition of the outer membrane, and whether it is possible to determine simple criteria for a more rapid assessment of cell quality than the time-consuming and expensive MPT.

We do not intend to review the literature on this subject, but the study was based on several observations we believe to be of particular importance. First, the adverse effect of inhibitory substances in the medium on the MPA level of cells (1) may be avoided by the use of the defined medium introduced by Stainer and Scholte (2). Using this medium it was possible to test the claim that the MPA of a culture may be increased by serial passage under constant growth conditions (3). Second, and equally important, was the finding that by the use of the rotary disintegrator (4) the cell walls of *B. pertussis* may be separated into two particulate fractions, the first of which may be sedimented by classical methods (5) at 10,000–12,000 × g and the second at 100,000 × g. This helped us to formulate a view concerning the identity of the stromata protective antigen (SPA) of Pillemer et al. (6). Preliminary results of these studies have been reported previously (7,8).

MATERIALS AND METHODS

Strains

The *B. pertussis* strains listed in Table 1 are from the MRC trial (9) isolated by the Public

Health Laboratory Service. The following were used as type strains: Serotype 1: strain 353/Z from Dr. N. Preston (Manchester). Serotype 1.2: CN 4132 from Lister Institute, London; strain 24 from Swiss Serum and Vaccine Institute (Bern, Switzerland) but originating from the Rijks Instituut voor de Volksgezondheid, Bilthoven, The Netherlands. Serotype 1.3: strain CN 5476 from the Hither Green Hospital, London. Serotype 1.2.3: strain CN 2992 from the late Dr. Holt and 366TS from Dr. J. Pekarek (Prague). *Bordetella bronchiseptica* strains 4424 (K 1,2; O 2); 835 (K 1, 3; O 3); and R1 (K 1, 3, 4; O 4) were from Dr. K. B. Pedersen, State Veterinary Laboratory, Copenhagen. The strains were stored as freeze-dried specimens or in liquid nitrogen as previously reported (10).

Culture Media

The defined medium used for shake flask, semi-continuous and continuous cultures was that of Stainer and Scholte (2) used as previously reported (10). The filter sterilized "vitamin solution" was stored at 4° C for a maximum of 1 week, and was added daily in the requisite volume to the basic medium necessary for 24-hour use. Although the pH in the fermentor was automatically controlled, the Tris buffer was not omitted from the medium, since it has been found that its presence facilitates the survival of the cells (10). To obtain successful growth from small inocula in this defined medium, the two following points were found to be critical:

- *Quality of water.* Only glass double-distilled water could be used. For water storage, plastic-ware/polypropylene containers were stringently avoided since a few minutes exposure to such material rendered the water unsuitable, a feature also found with the growth of gonococci in defined medium (11).
- *Cleanliness of glassware.* To remove from the glassware all traces of detergent it was necessary to rinse glassware thoroughly with tap water then soak it in 5% HCl for 5–24 hours. Finally the glass was thoroughly rinsed with distilled water.

Casein Hydrolysate Medium

This medium, either solidified with 1.8% w/v Oxoid Agar No. 3, or used in its liquid form with agar omitted, was a "Cohen-Wheeler"-type medium containing laboratory-made and purified casein hydrolysate as reported previously (10). Agar blood plates comprised the same solid medium supplemented with 10% v/v defibrinated horse blood (Wellcome Reagents Ltd).

Culture Methods

Since we have found that stainless steel is inhibitory for *B. pertussis* (see results), an all-glass fermentor vessel was used for the continuous and semicontinuous cultures. The fermentor consisted of a Sovirel 1,000 ml reaction vessel (Cat. No. 4.296-6O), cover with five ports (4.296-O3), stirrer bearing (4.588-O2), stirrer shaft (4.588-33) and teflon-coated stirrer rotor (4.588-97) (all supplied by V. A. Howe and Co., London SW6). The reaction vessel was modified by the addition of a glass side arm with a screw joint. The side arm served as a medium overflow and was positioned to maintain a working content of 500 ml in the vessel. A second identical outlet, positioned at the bottom of the vessel, was used for culture harvesting. The medium was pumped into the vessel (Vario-perpex pump, LKB) through one of the ports in the glass cover. To avoid contamination of inlet tubes, a series of safety bells was incorporated into the medium-flow line at a point just above the inlet port. A second series of safety bells was positioned immediately after the culture outlet port. All safety bells were ventilated by a continual flow of sterile air. All flexible connections were made of silicone rubber tubing (Esco-Rubber Ltd., London), and clamped where necessary with artery forceps. A T-piece was incorporated into the medium inflow line, to which a burette was connected for accurate medium-flow rate adjustment. A similar T-piece was incorporated into the effluent line to facilitate effluent culture sampling.

A constant, preselected pH was maintained, using a combined glass pH-electrode (Ingold, 465-35, Pye Unicam, Cambridge) that was inserted through one of the ports and connected to a Radiometer TTT2b titrator (V. A. Howe and Co., London SW6) that controlled the addition (Varioperpex pump, LKB) of the 1.0 N HCl. A galvanic type (Pb-Ag) pO_2 electrode was inserted through another port and was connected to a pO_2 meter (both electrode and meter supplied by Western Biological Equipment Ltd., Sherborne, England). The electrode was exchanged periodically for recalibration.

The vessel was heated by a 250W Philips infrared lamp controlled by a Thyristat TA5-O unit (Cressal Manufacturing Co. Ltd., Birmingham) with a contact thermistor ITT-M53W (Standard Telephone and Cables Ltd., Harlow, Essex) affixed to the side of the reaction vessel. The impeller speed was kept constant at 400 rpm. The air was sterilized, using a Stora MS Filter 681 (Camlab, Cambridge). Surface

aeration was preferred to sparging, since the latter produced serious foaming problems, and antifoams were either growth inhibitory (Polypropylene glycol 2000) or ineffective when used in long fermentor runs (MS Silicone antifoam A, Dow Corning). However, we found that the use of a large-diameter impeller with diagonal blades afforded sufficient agitation to maintain the air saturation above 10%, thus obviating the need for sparging. The vessel was unbaffled, but the inserted electrode disallowed vortex formation. During operation, the whole fermentor was housed in a dust-free cabinet. The fermentor was very simple; it presented no operational problems during runs lasting for several months.

This fermentor was used for semicontinuous cultures by sealing off the overflow side arm and harvesting the culture through the lower outlet. We have termed the interval between fresh medium addition and culture harvesting as a cycle. The culture was harvested before the end of exponential phase of growth, and a residue of at least 50 ml of the culture (e.g., 1/10 of the total volume) was left in the vessel as the inoculum for the next cycle. After harvesting, the vessel was immediately refilled with fresh medium to avoid any lag between successive growth cycles.

Small volumes of cultures and seed cultures for the fermentor were grown in 100 ml of the appropriate medium in 500 ml conical flasks. The necks were covered with loosely fixed caps, which were kept in position by cellotape. The flasks were incubated at 37° C on an orbital shaker (Type Rc-4-Sp, Kuehner, Basel, Switzerland) at 150 rpm with a radius of 50 mm. Under these conditions the oxygen exchange rate, as measured by the sodium sulfite method, was 20–40 μM O_2 hr^{-1}. Baffled flasks prevent growth from small inocula and were not used.

The flasks were inoculated with cultures grown and resuspended from agar blood plates or directly with liquid nitrogen frozen suspensions.

Formolization

The cultures were formolized by addition of 0.25% v/v formalin (37–39% formaldehyde solution, Analar, BDH) and left to stand for 24 hours (unless otherwise stated) at 37° C. The death rate under these conditions has been previously recorded (10). The cultures were then washed twice at 5,000 × g with phosphate buffered saline (PBS) (0.123 M NaCl, 0.01 M Na_2HPO_4, 0.0032 M KH_2PO_4,

pH 7.2) at 4° C, and resuspended in PBS containing 1/10,000 merthiolate.

During continuous cultures the collected effluent was formolized twice daily, or alternatively the formalin solution was added to the effluent automatically by a metering pump, which maintained a constant formalin concentration of 0.25%. The cultures were finally washed as above.

Disintegration and Fractionation of Cells

The method previously described was used (4). Briefly, B. pertussis was suspended in distilled water to a concentration of 20 mg dry weight of cells per ml and was pipetted in 7 ml lots into 28 × 100 mm thick walled Pyrex centrifuge tubes. The tubes were then cooled in a crushed ice-water bath and Ballotini glass beads No. 14 (Jencons Ltd., Hemel Hempsted, England) were slowly added until the level of liquid and Ballotini beads coincided (about 20 ml of beads). The cooled tubes were then inserted individually into the disintegrator and the ring stirrer was rotated at 4,000 rpm under constant cooling for 120–150 seconds. For semiquantitative recovery, the disintegrated slurry was diluted with an equal volume of water, transferred into a 25 × 60 mm glass tube that had been closed at one end with a sintered glass filter No. 1 and positioned inside a 35 × 120 mm centrifuge tube by means of a rubber collar. The liquid was then separated by low speed centrifugation for 1 minute. For quantitative recovery the Ballotini glass beads were washed several times.

The bead-free slurry was spun down at 3,000 × g for 20 minutes to remove unbroken cells. The resultant supernatant was respun for 30 minutes at 12,000× g to obtain the "cell-wall" fraction and again at 50,000–100,000 × g for 2 hours or 1 hour, respectively, to obtain the vesicular fraction. The two fractions were then washed at their respective speeds with distilled water, or with other solutes as necessary. It was imperative that disintegration occur immediately after harvest, and that all operations be performed at 0–4° C.

Centrifugation

Relative centrifugal forces referred to relate to the maximal diameter of the rotors used: SS–34 and GSA in the Sorvall RC2–B centrifuge, and (in excess of 48,000 × g) the 8 × 35 aluminum angle head rotor in the MSE Superspeed 65 ultracentrifuge.

Gradient Centrifugation

Metrizamide (Nyegaard and Co., Oslo, Norway) dissolved in 0.05M Tris-HCl, pH 7.8, was layered into 35 ml tubes in the following sequence: 1 ml cushion of 65%; 4 ml of 50%; 5 ml of 30%; and 6 ml of 15% (all w/v). The tubes were left to stand overnight at 4° C. The sample (1 ml sample plus 1 ml 15% Metrizamide solution) was applied to the top of the gradient before centrifugation, and the remaining space in the tube was filled with a light paraffin oil. The tubes were spun in the MSE 8 × 35 ml aluminum angle head at 26,000 rpm for 2 hours, followed by 8 hours at 20,000 rpm at 15° C. The fractions (1 ml) were collected by piercing the tubes, and the refractive index of each was determined. The respective densities were then calculated using the formula p = 3.350c − 3.462 (12).

Chemical Determinations and Extractions

Deoxysugars from both vesicles and whole cells were liberated by 0.2 N HCl or 0.2 N H_2SO_4 hydrolysis at 100° C for 10 minutes with subsequent separation on Dowex 1, carbonate, 200–400 mesh (13). The deoxysugars were determined using the 2-thiobarbituric acid assay (14), and the spectral maxima of the chromogens formed were determined with a Beckman DB GT spectrophotometer. 2-Keto-3-deoxyoctonate (Sigma) was used as a standard.

Since it has been found that this procedure also liberates agglutinogens from the outer membrane (see Results) the method was suitably modified. The hydrolysis was routinely performed by mixing an equal volume of 0.2 N HCl with either the cell suspension (10–20 mg dry weight cells per ml) or isolated outer membrane vesicles and incubating the mixtures overnight at 37° C. During this procedure the pH of the mixtures ranged between 1.5–3.0. The mixture was then neutralized with NaOH, spun down at 6,000 × g for 20 minutes at 4° C, and cooled to 0° C. An equal volume of acetone (Analar, BDH) precooled to −20° C was added slowly to the supernatant. The mixture was then left to stand overnight at −20° C then spun down at 6,000 × g for 20 minutes at −5° C. The acetone precipitate was dissolved in a small volume of cold distilled water (1/10–1/20 of the original volume), centrifuged at 100,000 × g for 2 hours at 4° C to remove any particulate matter, and finally freeze-dried. *Phenol LPS* was extracted by the phenol-water technique (15). The LPS was collected from the phenol-free dialysis retentate by centrifugation (100,000 × g for 2 hours), washed with water, and freeze-dried.

Nitrogen content was determined by the micro-Kjeldahl method as described by Kabat and Mayer (16).

Reagents

Trypsin (type 1, Sigma) was dissolved in cold 0.01 N HCl prior to use. Cytochrome c was type III (Sigma). Pillemer's Stromata Protective Antigen was from a sample used in the MRC trial (17), which was received as a gift from Dr. F. Perkins (WHO, Biologicals, Geneva). The sample was labeled SPA, 2nd batch.

Clostriduim welchii α-toxin was from Wellcome Reagents Ltd. Alhydrogel was supplied by Superflos, Copenhagen.

Chromatography

Thin layer chromatography of amino acids was carried out on washed DC-Cellulose F (Merck) plates (18). Dansylation of the pooled membrane proteins was by the method of Gray (19) using Cheng Chin polyamide sheets (BDH). Descending chromatography with Whatman 3MM paper in butan-1-ol-pyridine-water (6:4:3) was employed for sugars obtained from Dowex 1-carbonate columns (13), and the spots were developed using Warren (20) and p-anisidine HCl sprays.

Column Chromatography

Pharmacia K25/45 and K25/100 columns were used for DEAE-Sephadex A–25 and Sephadex G–25 (fine), respectively. The DEAE Sephadex A–25 was equilibrated and the column eluted with 0.1M Tris-HCl buffer pH 8, with 0.002% v/v Hibitane (ICI) added as a preservative. A linear sodium chloride gradient was pumped from a gradient mixer containing 200 ml of the elution buffer in the first compartment and 200 ml M NaCl in the elution buffer in the second compartment. Fractions eluted by the NaCl gradient were freeze-dried, dissolved in a small volume of water, and desalted using Dowex 50W, 200–400 mesh, H+ form, contained in a 25 × 300 mm glass column, with distilled water as the eluant.

The Sephadex G–25 (fine) column was equilibrated and eluted with 0.1 M ammonium bicarbonate containing 0.05% sodium azide. Elution patterns were monitored by a Uvicord (LKB 8300) at 280 nm and the fractions (6 ml) were collected on an LKB UlroRac. The fractions were tested for

sugar content by the phenol-H_2SO_4 method (14). Column chromatography was performed at 4° C.

Sugars positive in the 2-thiobarbituric acid assay (14) were chromatographed on Whatman cellulose, standard grade, in a 12 × 270 glass column, equilibrated and eluted with 85% v/v acetone in water. Fractions (0.5 ml) were eluted and tested in the deoxysugar test.

Antisera

Rabbits were immunized with antigens in incomplete Freund adjuvant by two 1-ml intramuscular injections 3 weeks apart. The antigen content was 2.5 mg per ml. This basic course was followed by a total of six intravenous injections of alum-precipitated antigen (containing 2.5 mg antigen per ml and 10% alum), the injections being applied twice weekly in volumes increasing from 0.05 ml to 0.4 ml. The animals were bled 10–12 days after the last injection.

The factor sera were either commercial sera (Wellcome Reagents Ltd.) or were prepared in our laboratory by Andersen's method (21). Rabbits were immunized with strains 353/Z (serotype 1) or strains CN 2992 or 366TS (both 1.2.3). The sera of rabbits immunized by serotype 1 strain were adsorbed only by cells heated for 2 hours at 120° C. The sera of rabbits containing antibodies against factors 1, 2, 3 were adsorbed by suspensions of cells grown in casein hydrolysate medium (see Results) and displaying agglutinogens 1, 2 (to obtain factor 3 serum) or agglutinogens 1, 3 (to obtain factor 2 serum). The sera were finally adsorbed by heated cells (2 hours at 120° C). Adsorptions were performed at 37° C for 1 hour followed by an overnight incubation at 4° C. The sera were tested with cell suspensions of known agglutinogen content, and finally diluted to give a maximum agglutination titer of 1/320–1/640.

Horse sera (RX5259B/36 and RX5360/36) raised against disintegrated *B. pertussis* cells were as previously reported (10). *B. bronchiseptica* sera were obtained by immunizing rabbits with strains 4424, 835, and Rl. *B. parapertussis* serum was a pooled rabbit serum obtained from Wellcome Reagents Ltd. All of the sera except the factor sera were used unadsorbed.

Agglutination

The antigen was formolized, washed with PBS, and resuspended to a density of 1 mg dry weight of cells per ml in PBS containing 1/10,000 merthiolate.

Serial twofold dilutions of the sera were made in plastic trays (U-shaped wells, Linbro, IS–MRC–96, 0.05 ml per well), using Microtitrator diluters. Fifty microliters of standardized antigen was then added to each well, after which the trays were sealed with self-adhesive membranes, mixed by shaking, incubated for 1 hour at 56° C in a water bath, and finally left overnight at room temperature before being read under a Nikon plate microscope with oblique illumination.

Agglutination Inhibition Test

Twenty μl volumes of the test solution (containing 5–200 μg of the inhibitor per ml of PBS) were added to the last three dilutions of sera which had shown agglutination in the preliminary agglutination test. Twenty μl of the PBS was added to all remaining wells. Control serum dilutions with the inhibitor omitted were always run in parallel. The plates were sealed with self-adhesive membranes, shaken, and incubated for 1 hour at 37° C in a water bath. Standardized antigen was then added and the trays were resealed, shaken, and incubated as above. It was uneconomical to try to inhibit all of the dilutions of the sera shown in Table 3.

Crossed Immunoelectrophoresis

This was carried out according to Smyth et al. (22) on 5 × 5 cm glass slides using HGR agarose (Uniscience Ltd., Cambridge) with 0.06 M Tris-HCl buffer, pH 8.6, instead of barbiturate. The slides were run for 70 minutes at a potential difference of 70 V in the first direction and overnight at 20–30 V in the second direction. Countercurrent immunoelectrophoresis (CCIE) was also carried out on 5 × 5 cm slides coated with agarose. By means of a template, series of 3 mm wells 2 mm apart were cut into the gel. The slides were subjected to a potential difference of 70 V for 30 minutes. This afforded us a rapid screening of antigens and antisera.

Animal Tests

Mouse-protection test (MPT). The whole cell or cell fractions were suspended in PBS and tested as previously reported (10) (except that, unless stated, 4 groups of mice were used (10–16 mice per group, 14–16 g), and injected with 1/1, 1/3, 1/9 or 1/27 dilutions of the preparation under test, to decrease fiducial limits (D. A. Field and P. Novotny, unpublished). Each sample was subjected to at least two independent tests, which were accepted as valid only when the groups covered by the dilution range

showed $>50\%$ and $<50\%$ survivors. Strain 18323 was used as the challenge and, unless otherwise stated, was stored as a single batch in liquid nitrogen. The reference vaccine used was British Standard 66/84, and Scoffield outbred mice were used throughout. The dose level was adjusted for the two sexes (10).

The results were computed using a parallel line probit analysis program, with inputs in units of μg dry weight of bacteria with experimental cell suspensions, or ml with blended vaccines. The relative potencies were expressed as both ED_{50} and international units (IU) per μg or ml. From a series of experiments which measured the optical density of several strains grown under various conditions (10), it was found that 60 WHO opacity units corresponded to 1.0 ± 0.25 mg dry weight of cells. A similar value has been determined elsewhere (23). For convenience, therefore, 1 mg of dry weight of whole cells may be regarded as the total human dose. Dry weight determinations were performed as previously reported (10).

Toxicity for embryonated eggs. Eleven-day-old embryonated eggs (White Leghorn, "Kimber") incubated at $37°$ C received 0.1 ml intravenous injections of fivefold dilutions of the test sample (10 eggs for each dilution). Deaths were recorded after 24 hours. Since embryonated eggs are extremely sensitive to *B. pertussis* intracellular toxin, all preparations had to be heated for 30 minutes at $56°$ C before use.

Electron Microscopy

Negative staining. Samples were suspended in distilled water mixed with an equal volume of 1.5–3.0% w/v sodium phosphotungstate, pH 6.5, and observed on Formvar carbon-coated, 400 mesh copper grids.

Ultrathin sections. Whole-cell and disintegrated-cell samples were fixed by the immediate addition of 1/10 volume of 5% v/v acrolein, 0.25% v/v glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.5 (all obtained from EM Scope Ltd., London SW4), spun at $5,000 \times g$ for 5 minutes, and the pellet resuspended in acrolein-glutaraldehyde for 3–24 hours. This technique, described by Burdett and Murray (24), was found to be the only technique suitable for the study of *B. pertussis*. Postfixation did not appear to be as critical. However, the recommended postfixation in 1% OsO_4 in 0.05 M cacodylate buffer, pH 7.5, for 1 hour at room temperature gave the best results. The pellets were

dehydrated using ethanol, embedded in Araldite (Ciba Ltd), and the sections cut on an LKB Ultratome and mounted on 200-mesh Formvar coated-copper grids. Sections were then stained with 2% w/v uranyl acetate for 10 minutes. Philips EM 200, 300 or 301 electron microscopes were used.

RESULTS

Growth of Fresh *B. pertussis* Isolates in Defined Medium

The suitability of chemically defined media for the growth of fresh isolates of *B. pertussis* is less well documented than for the more "adapted" laboratory strains. For this reason we have undertaken a study of the growth rates, final yields, agglutinability, and protective activity of shake flask cultures of recent *B. pertussis* isolates in defined medium. The strains studied were isolated by the Public Health Laboratory Service (PHLS) (9) and deposited in our culture collection. Although differences occurred between the growth rates of the freshly isolated strains, as was the case with the "adapted strains" (doubling times ranged between 5–9 hours), the final densities obtained at the end of the exponential phase were comparable (approximately 1.5 mg dry weight per ml), and most of the cultures showed some level of MPA (Table 1). However, five strains (Nos. 222, 565, 726, 799, and 1064) showed a level of protective activity similar to that of vaccine production strains grown under the same conditions.

From Table 1 it is further apparent that the strains designated as serotype 1.2 by the PHLS grew as serotype 1.2.3 in the defined medium. Similar behavior was observed with 8 collection strains, some of which were used as serotype 1.2 in the preparation of factor sera for agglutinin adsorption. To grow these strains as serotype 1.2 it was necessary to use casein hydrolysate media, which inhibited the formation of factor 3. Only one laboratory strain (CN 4132) showed no agglutination with factor serum 3 when grown in defined medium. It appears, therefore, that the serotype 1.2 of *B. pertussis* is rather rare, and that the majority of strains previously regarded as serotype 1.2 are, under certain growth conditions, capable of growing as serotype 1.2.3.

Table 1 also shows that among the 28 strains randomly selected from a field trial, the growth of two strains (50 and 596) was accompanied by a chocolate-like discoloration of the medium, charac-

teristic of *B. parapertussis,* which implies that the incidence of this organism in Britain may be higher than usually assumed. Color-free defined medium facilitates recognition of this species.

From these preliminary observations, and in accordance with a previously published report (25), we have concluded that the Stainer-Scholte type of defined medium is suitable as a general growth medium for physiological studies in *B. pertussis.*

Fractionation of *B. pertussis* Cells

Disintegrated cells were separated by centrifugation into a 12,000 × g fraction, a 50,000-100,000 × g fraction, and the subsequent final supernatant, which was always alkaline (pH 8.6–9.0). The 50,000–100,000 × g fraction was relatively homogeneous and consisted predominantly of vesicles of about

Table 1. Relative Potencies of "Wild" Strains of *B. pertussis* Grown in Defined Medium [a]

Strain PHLS No.	Declared Serotype	mg Dry Weight per 12 I.U.[b]	Comment
50	1.2.3	—	Brown pigment
90	"	4.077	
140	"	1.722	
180	"	2.664	
231	"	3.121	
253	"	3.917	
370	"	4.757	
338	"	9.990	
564	"	18.163	
596	"	—	Brown pigment
695	"	1.364	
731	"	1.698	
736	"	4.647	
989	"	3.027	
997	"	5.708	
1046	"	3.770	
276	1.2	NT	Grew as serotype 1.2.3
825	"	2.594	
158	1.3	6.889	
222	"	0.447	
243	"	4.206	
348	"	3.632	
565	"	0.410	
634	"	1.297	
690	"	1.715	
726	"	0.380	
799	"	0.696	
1064	"	0.622	

[a] Shake flask cultures (third subculture) were formolized at the end of exponential growth.

[b] Each vaccine was tested at three dose levels and the results expressed as the arithmetic mean of two independent determinations. The 18323 challenge strain used was from a different frozen batch than that referred to elsewhere in this paper.

100 nm (Fig. 1A). When this fraction was applied to a Metrizamide density gradient, 70–82% of the total nitrogen content of the sample was concentrated in a strong and clearly visible band with a buoyant density of 1.218–1.224. This value is in agreement with the buoyant density of the outer membrane of *Salmonella typhimurium* (26). Adhesion of disrupted inner membranes to the collapsed cell walls (Figs. 1B, C) facilitated separation of the outer layer in a relatively pure form, with contamination usually restricted to a few pili. The vesicles comprise about 9.9% of the dry weight or 9% of the total nitrogen content of the cell (Table 2). It is important to stress, however, that after disintegration no defects in the surface are apparent (Figs. 1B, C) as some residual outer membrane remains bound to the cell wall. We conclude that during the mechanical removal of the substantial part of the outer membrane, the residuum possesses some form of "self-repair property," its fluidity allowing it to remain stretched over the cell wall surface.

As may be seen from Table 2, most of the mouse protective activity is bound to the vesicular fraction, and the remaining activity is located in the cell wall fraction. However, this division depends on the duration of disintegration. The optimal time for obtaining the maximum MPA in the vesicular fraction was found to be 120–150 seconds. After 20–40 seconds of disintegration, the cell wall fraction may contain up to 30% of the total MPA, whereas when disintegration time exceeds 240 seconds the total amount of MPA decreases, most probably as a result of surface denaturation. One may conclude, therefore, that the MPA of the *B. pertussis* cell is bound to the outer membrane. The ED_{50} of the heated outer membrane vesicles for embryonated eggs ranged between 200–400 μg dry weight. The outer membrane vesicles therefore represent the natural form of *B. pertussis* endotoxin.

Fractionation of the Outer Membrane

When the vesicles were hydrolyzed with 0.2 N H_2SO_4 or 0.2 N HCl at 100° C for KDO content determination (13), compounds positive in the 2-thiobarbituric acid assay (see below), plus a considerable amount of acetone precipitable solids, were liberated. None of these components was active in the agglutination inhibition test. However, when the method of hydrolysis was suitably modified (see Methods) and performed at 37° C, the acetone precipitable moiety inhibited the agglutination of
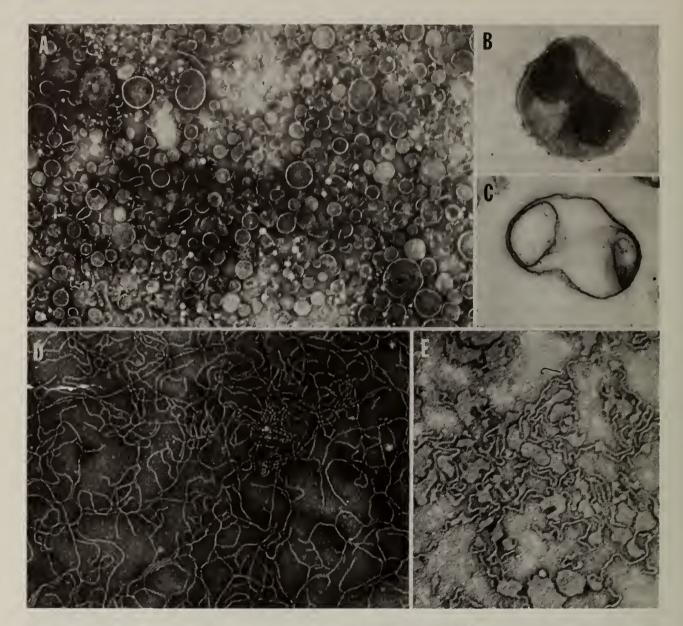
Figure 1. (A) Outer membrane vesicles of *B. pertussis*. (B) Disintegrated cell showing adhesion of internal membrane to cell wall ×30,000. (C) As in (B) Thin section ×54,000. (D) Phenol-LPS of *B. pertussis* ×91,500. (E) Conversion of outer membrane vesicles to LPS fibers after 2 min. exposure to phenol ×59,000. Specimens in A, B, D, and E were negatively stained.

cells of serotype 1.2.3 by factor sera (Table 3), provided that the substance originated from cells that displayed all three serotype factors. When the substance was extracted from cells of serotype 1.2, only agglutination by factor sera 1 and 2 was inhibited. Corresponding inhibition patterns were observed with cells of other serotypes. The hydrolysis yielded maximum results after 14–18 hours at 37° C. Mild acid hydrolysis evidently removes part of the outer membrane of *B. pertussis*, which is precipitable by acetone and inhibits agglutination. We have termed this *crude agglutinogen*.

Further investigation showed that whole cell suspensions subjected to the same treatment also produced this crude agglutinogen with final yields of up to 8.5% by weight (see below). Isolated vesicles gave lower yields of crude agglutinogen than expected (3–3.5%). This may be due to more rapid decomposition by the acid, since the amount of free acid present during their hydrolysis was higher.

When the crude agglutinogen was applied to a DEAE-Sephadex A–25 column, the pattern shown in Figure 2 was obtained, irrespective of serotype. The pooled fractions were tested for agglutination

Table 2. Distribution of Disintegrated *B. pertussis* Cells by Centrifugation

	Percent of whole cells		Percent of Mouse Protective Activity [a]
	by dry weight determination	by N determination	
Sediment at 12,000 × g.			
Cell walls + plasma membranes, some outer membrane vesicles	29.8	30.5	3–13%
Sediment at 100,000 × g.			
Predominantly outer membrane vesicles	9.9	9.0	50–76%
Supernatant at 100,000 × g.	56.8	62.0	None [b]

[a] The mouse-protective activity is expressed as a percentage of the total mouse-protective activity of whole cells before disintegration and is related to volumes of fractions obtained. The % relate to lower and upper limits of all preparations so far tested.

[b] This fraction is highly lethal for mice unless heated at 56°C for 30 minutes.

inhibition. Material inhibitory for agglutination by factor sera 2 and/or 3 (termed agglutinogens 2 and 3 respectively), always coincided with the first peak (P1), whereas the material inhibitory for agglutination by factor serum 1 was retained within the column and was only eluted by the application of a sodium chloride gradient (Pool 3, Fig. 2). When crude agglutinogen prepared from cells of serotype 1 was applied, the first peak, although present, showed no agglutination inhibition.

When material from the first peak (P1) was precipitated by acetone and applied to a Sephadex G–25 (fine) column, two peaks were usually recorded. The first (P11, Fig. 3) contained agglutinogens 2 and/or 3. The second, when present, was inactive. By the chromatographic methods used it was impossible to separate agglutinogens 2 and 3. Their individual molecular weights are smaller than that of cytochrome C; in fact, the whole crude

agglutinogen will dialyze through unstretched Visking cellophane tubing.

When hydrolyzed with 6 N HCl, material from P11 was found to contain the usual protein amino acids, with the exception of cysteine/cystine. The scoring of the amino acids on TLC chromatograms was as follows: Arg + + +; His +; Lys + +, Gly + + + +; Ser + + +; Asp + + +; Glu + + +; Ala + +, Thr + +; Val + + +; Ile + +; Leu +; Phe +; Met trace; Pro trace. The Pro concentration was especially low. Alkaline hydrolysis for the detection of Trp was not performed. After dansylation, the only free terminal amino-group detected was the epsilon amino-group of lysine. The P11 material also showed a weak reaction in the phenol-H_2SO_4 test, but the identity of the sugar component was not investigated.

The pooled material from the DEAE Sephadex A–25 column, which inhibited agglutination by

Table 3. Test on Agglutination Inhibition

Substance Tested for Inhibition [a]	Serum	Agglutination Titer [b]						
		4	8	16	32	64	128	256
None (PBS control)	Factor serum 1	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+	−
	" " 2	+ + + +	+ + + +	+ + + +	+ + + +	+ +	−	−
	" " 3	+ + + +	+ + + +	+ + + +	+	−	−	−
Agglutinogen obtained at 100°C	Factor serum 1	+ + + +	+ + + +	+ + +	+ +	+	−	−
	" " 2	+ + + +	+ + + +	+ +	+ +	+ +	−	−
	" " 3	+ + + +	+ + +	+	−	−	−	−
Agglutinogen obtained at 37°C	Factor serum 1	+ +	+ +	±	−	−	−	−
	" " 2	+	−	−	−	−	−	−
	" " 3	−	−	−	−	−	−	−
Phenol LPS	Factor serum 1	+ + + +	+ + + +	+ + + +	+ + + +	+ +	−	−
	" " 2	+ + + +	+ + + +	+ + + +	+ + + +	+ +	−	−
	" " 3	+ + + +	+ + + +	+ +	+	−	−	−

[a] Twenty μl of samples under test (5 mg per ml PBS) were added to each well. All fractions originated from cells of strain CN 2992 (serotype 1.2.3).

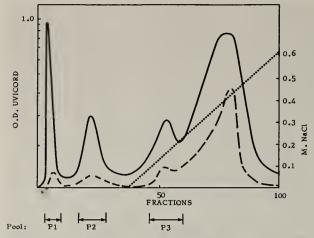[b] *B. pertussis* cells, serotype 1.2.3 were used as antigen.

Figure 2. Elution pattern of crude agglutinogen (100 μg sample) from DEAE Sephadex A–25 column. ———— Uvicord record, – – – – relative sugar content of fractions, - - - - NaCl concentration.
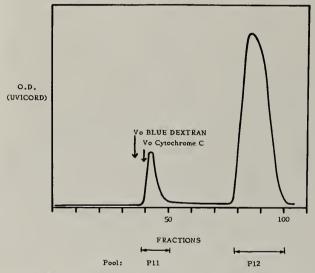

Figure 3. Elution pattern of pool P1 when applied to Sephadex G–25 (fine) column.

factor serum 1 (P3, Fig. 2), usually produced the pattern shown in Figure 4 when desalted and applied to a Sephadex G–25 (fine) column. Agglutinogen 1 was eluted in pool 3 (P33). A flow diagram of the separation sequence of the crude agglutinogen is shown in Table 4.

Analysis of Crude Agglutinogen Fractions by Crossed Immunoelectrophoresis

This technique showed that the material from pool P11, which usually contained agglutinogens 2 and 3, is complex. When tested using rabbit or horse sera raised against whole cells, P11 contained 9 antigens, labeled P111–P119 (Fig. 5 and Table 4). Horse sera were particularly useful in these tests

(Fig. 6A). These antigens, which were found to be present in all of the *B. pertussis* serotypes, did not react with sera raised against whole cells of *B. bronchiseptica,* sera raised against *B. pertussis* phenol-LPS, or with *B. pertussis* factor sera 1, 2, and 3. They are therefore, unrelated to the agglutinogens. However, antigens P112, P113, and P114 formed precipitation lines with pooled serum raised against *B. parapertussis* (Fig. 6B).

On the other hand, of the fractions obtained from pool P3 (Fig. 4, P31–P33), only P31 showed any reaction. This material, labeled P311, moved more anodically (Figs. 5, 6C) than the other antigens, and reacted with sera raised against whole cells of *B. pertussis, B. parapertussis* and *B. bronchiseptica,* serotypes K 1, 2–O 2; K1, 3–O 3 but not with *B. bronchiseptica* serotype K 1, 3, 4–O 4. No reaction with *B. pertussis* factor 1 serum was observed. It reacted, however with sera raised against fibrillar, phenol-extracted *B. pertussis* LPS (see below). This material therefore shares a common determinant with phenol LPS, and its position in crossed immunoelectrophoresis suggests that it corresponds to precipitate No. 7 in the map of antigens obtained from *B. pertussis* by Hertz et al. (27).

Biological Properties of Crude Agglutinogen

Injected with incomplete Freund adjuvant, crude agglutinogen prepared at 37° C induced the formation of rabbit antibodies that agglutinated *B. pertussis* cells at comparatively low titers (i.e. 1/2048 as compared with titers of 1/20,000–1/40,000 when whole cells were used). Such sera usually reacted very weakly in CCIE. Crude agglutinogen showed no protective activity in the mouse-protective test.

Fractions obtained from Sephadex G–25 columns were unable to produce agglutinating antibodies in rabbits and their sera did not react in CCIE. It is possible, therefore, that the various components that constitute the crude agglutinogen complement each other antigenically.

It has been shown, therefore, that by mild acid hydrolysis a substantial part of the outer membrane of *B. pertussis* can be split into various subunits with molecular weights of approximately 10,000. Some of these precipitate with their corresponding antisera, while the biological activity of others may only be assessed by agglutination inhibition. The latter appear to be haptens, carrying determinants for agglutinogens 1, 2, and 3.
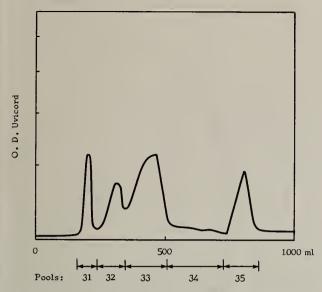
Figure 5. Precipitation pattern of *B. pertussis* outer membrane antigens in crossed immunoelectrophoresis. S denotes the application origin of the antigen.
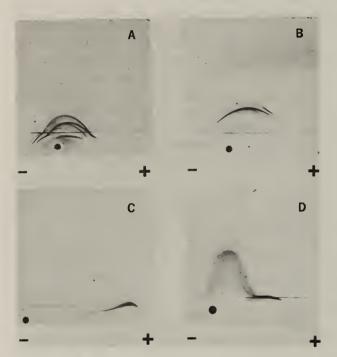
Figure 6. Crossed immunoelectrophoresis of *B. pertussis* outer membrane antigens. (A) Precipitation pattern of proteins P112, P113, P114, P115, P117, and P118 from pool P11 (100 μg sample) from strain CN 2992 grown as serotype 1.2.3 when reacted against antipertussis horse serum RX5258B/36. (B) As in (A) but reacted against pooled rabbit *B. parapertussis* antiserum. (C) Precipitation pattern of antigen P311 from pool P31 (100 μg sample) with rabbit serum raised against outer membrane vesicles. (D) Precipitation pattern of freshly prepared phenol-LPS (300 μg sample) with antipertussis horse serum. Black dots denote the application origin of the antigen.

The eluates were freeze-dried, and the residues were dissolved in cold 95% acetone (the crude agglutinogen does not dissolve under these conditions). The sugars were chromatographed on paper (butan-1-ol-pyridine-water, 6:4:3) and the sugars were detected using the Warren and p-anisidine
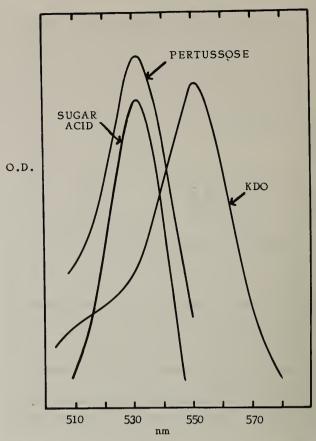
Figure 7. Absorption spectra of *B. pertussis* deoxysugar chromogens in the 2-thiobarbituric acid assay. With the method used the absorption maximum of commercial 2-keto-3-deoxyoctonate (KDO) was identical to that of KDO obtained from *E. coli*.

sprays, which produced pink and brown-gray spots, respectively. The pertussose remained close to the origin, whereas the sugar acid moved slightly faster (R_{Rha} 0.04 and 0.24 respectively). However, neither of them moved as fast as any known dideoxyhexoses of bacterial origin (28). Both were very labile, even when stored at −100° C.

The deoxysugar acid was chromatographed on a cellulose column, and several peaks positive to 2-thiobarbituric acid assay were eluted (Fig. 8). The first peak was produced only when a freshly prepared sample was applied, either decreasing or finally disappearing if the sample had been stored for more than a week at −100° C. When present, this peak showed the strongest color reaction. The material in the other peaks was more stable. The chromogen showed a maximum of 532 nm, with a very narrow range for the first peak, but slightly wider ones with the other peaks. However, the maximum was never in the region of 548 nm, the wavelength at which authentic KDO gave a typical
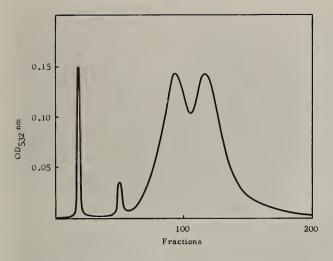
Figure 8. Elution pattern of *B. pertussis* deoxysugar acid (200 mg sample) from a cellulose column. Fractions were tested in the 2-thiobarbituric acid assay and extinctions measured at 532 nm.

maximum (Fig. 7). It is not known whether the deoxysugar acid fraction is a single component that rapidly decomposes or a mixture of several components.

None of these sugars could inhibit the agglutination of *B. pertussis* by factor sera.

Although KDO has been reported in *B. pertussis* LPS (29), the evidence for its presence was obtained after an unusually vigorous hydrolysis (2 N HCl for 2 hours) and the absorption maximum of the 2-thiobarbituric acid assay chromogen was not given, making comparison of the results impossible.

Lipopolysaccharide (LPS) in the Outer Membrane Vesicles

Phenol extracted *B. pertussis* lipopolysaccharide consists of long fibers 7–8 nm in diameter, which are occasionally branched and sometimes take the form of rings or loops (Fig. 1D). We have obtained about 3% of the dry weight of whole *B. pertussis* cell in the form of LPS. Isolated outer membrane vesicles adopt this fibrous form after 1–2 minutes exposure to phenol (Fig. 1E), with complete transformation occurring after longer treatment. After the phenol treatment of vesicles the fibrous LPS is sedimented from the aqueous phase at 100,000 × g with final yields usually around 13–15% by weight. LPS is unable to inhibit the agglutination of *B. pertussis* cells by factor sera 1, 2, and 3 (Table 3). When whole cells were extracted with phenol, the phenol (organic) phase could, after dialysis against water, be separated into a "rubbery" portion and a fine, granular precipitate. The rubbery phase was

inactive, but the granular phase, although insoluble in PBS, inhibited the agglutination of *B. pertussis* cells by factor sera 1, 2, and 3. Hydrolysis showed an amino acid content similar to that of crude agglutinogen, with the epsilon-dansyl amino group of lysine again being the only free terminal amino group detectable. It is probable, therefore, that phenol treatment removes the crude agglutinogen components from the LPS and transfers them to the phenol (organic) phase. LPS is always totally inactive in the mouse-protective test.

In CCIE the fibrous LPS always reacted with sera raised against whole cells, outer membrane vesicles (such sera were usually the most active), crude agglutinogens, and fibrous phenol LPS (the latter two sera usually being the weakest). In crossed immunoelectrophoresis the phenol LPS migrated about 75% of the way across the slide in the first direction, and, when run in the second direction, it produced a complex precipitation line (Fig. 6D). However, this occurred only when freshly prepared LPS was tested; when preparations stored at room temperature for several months were used, several distortions were observed, particularly at the start and at the anodal end of the line.

Effect of Trypsin on the Outer Membrane

"Cylindrical molecules" 120 × 120 Å have been observed on the cell surface after short exposure of *B. pertussis* cells to trypsin (30). Our studies with outer membrane vesicles confirm this observation. Figure 9C shows "ring structures" on the outer membrane that are reminiscent of those found in the gonococcus (31). Longer exposure to trypsin results in the removal of these structures (Fig. 9D), leaving only fine sheets of outer membrane, which comprise 14.6% by weight of the original vesicles. Similar figures have been obtained after phenol treatment of outer membrane vesicles. Indeed, remnants of vesicles remaining after trypsin treatment can be converted into fibers by phenol treatment. This shows that in its natural form LPS is a sheet-like lattice. After 2 minutes exposure to trypsin, cells or vesicles retain up to 30% of their original protective activity; treatment for 10–30 minutes destroys the MPA completely. The fragments obtained by trypsin treatment have not yet been investigated with crossed immunoelectrophoresis.

Identification of Pillemer's Protective Antigen

Pillemer's antigen used in the MRC trial (17) was applied in three monthly doses of 1 ml each.
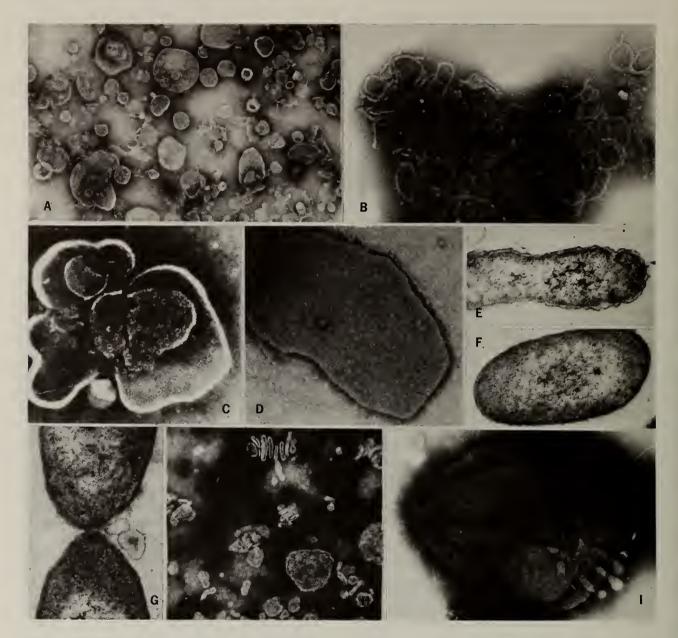
Figure 9. (A) Pillemer's SPA after enzyme treatment and sedimentation (see Methods) ×43,750. (B) Pillemer's SPA; conversion of vesicles into LPS fibers by phenol treatment ×142,500. (C) Outer membrane vesicle after 2 min trypsin treatment ×105,000. (D) Outer membrane vesicle after 20 min trypsin treatment ×99,000. (E) Thin section of *B. pertussis cell*, CN 2992, serotype 1.2.3 from an unbalanced culture showing low agglutination titer with factor serum 3 ×54,000. (F) The same as in (C), but from a balanced culture showing full agglutination pattern. Thin section ×40,500. (G) As in (F), showing liberation of outer membrane vesicles during cell division. Thin section ×60,000. (H) Outer membrane vesicles sedimented at 50,000×g from supernatants of *B. pertussis*, CN 2992, grown in semicontinuous culture at 24° C ×82,500. (I) *S. typhimurium* LT2 disintegrated in the rotary disintegrator. Liberation of outer membrane vesicles before complete removal of pili ×51,000. Specimens in (A), (B), (C), (D), (H), and (I) were negatively stained.

In 1974 we retested a sample from this trial in the mouse-protective test and found that 12 international protective units were contained in 8.2 ml. Thus after 21 years of storage the antigen still showed some protective activity, although the actual drop in MPA is difficult to assess since the potencies in the MRC trial were based on a different standard.

The antigen consisted of a sonicate of *B. pertussis* cells whose protective activity was adsorbed onto human red blood cell stromata. To remove the red blood cell stromata, we treated a sample of the antigen with α-toxin of *C. welchii* (20 ml antigen was supplemented with $CaCl_2$ to 0.002 M, mixed with 0.25 combining unit of α-toxin and incubated for 2 hours at 37° C and overnight at 4° C), sedimented the antigen at 60,000 × g for 2 hours, and washed the sediment twice with distilled water under the same conditions. Negative staining showed that the antigen consisted of vesicles similar to those obtained by disintegration of cells (Fig. 9A). When these vesicles were treated with phenol they were transformed into typical LPS fibers (Fig. 9B).

It appears, therefore, that Pillemer's antigen consisted of outer membrane vesicles adsorbed to human red blood cell stromata. To confirm this, we adsorbed our vesicle preparation to human red blood cell stromata following Pillemer's formula (6). [Protocol: disintegrated cells (Strain PHLS 731) were diluted in distilled water to a concentration equivalent to 9 mg dry weight of cells per ml. Nineteen parts of this suspension were mixed with one part of Eagle's solution and the pH was corrected to 7.0 with a few drops of M KH_2PO_4. The mixture, after being incubated overnight at 4° C, was centrifuged at 3,000 × g for 2 hours. To 30 ml of the supernatant 1 ml of human red blood cell stromata was added, the mixture was incubated for 3 hours at 37° C, and centrifuged for 2 hours at 3,000 × g at 0°C. The sediment was resuspended in glycine-phosphate buffer to a final volume of 300 ml. It contained 10 IU per ml with an MPA recovery of approximately 60% as compared with the MPA of the cells before disintegration.] A similar preparation, prepared with rabbit red blood cell stromata, was antigenic for rabbits (10).

SEMICONTINUOUS CULTURE OF
B. pertussis

Reports suggesting that serial passaging of *B. pertussis* leads to increased levels of MPA (3,32) indicate that the number of generations possible during a single batch culture is insufficient to attain such high levels. As our preliminary experiments showed, bacteria whose surface structure composition is highly dependent on environmental conditions may need a relatively longer period to switch from one state of physiological growth to another (33). We studied this possibility by using semicontinuous cultures, which enabled us to extend the

exponential growth phase far beyond the limits possible with batch culture, and at the same time to obtain enough cells at any given time for analysis. In this respect it had an advantage over continuous culture, in which the yield of cells is constant but minimal at any given time. A theoretical comparison of the principles involved in semicontinuous and continuous cultures has been made elsewhere (34). In our semicontinuous cultures, a proportion of the culture was harvested and replaced by an equal volume of medium of identical composition before the end of the exponential growth phase was reached (Fig. 10). The volume of culture exchanged depended on analysis requirements. Cultures were grown in this fashion for several months at a time, the total experiment lasting for 9 months. Defined medium was used throughout.

Effect of Growth Temperature on the Composition of the *B. pertussis* Outer Membrane

At the beginning of our experiments, criteria for balanced growth of *B. pertussis* were unknown. To elucidate this situation we concentrated on study of the composition of the outer cell membrane, espe-
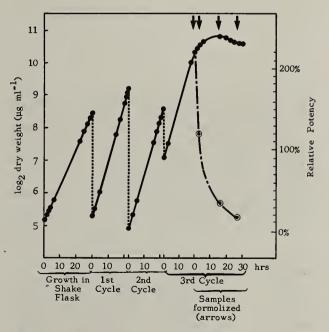
Figure 10. Semicontinuous growth of *B. pertussis*, 366TS. Formolization experiment. ● = dry wt, μg/ml. The relative potencies ⊙— - —⊙ were calculated on the assumption that 1 mg dry weight of cells should contain 12 IU (100% potency). The corresponding weights of cells containing 12 IU were: 0.4 mg (formolized at time 0), 0.734 mg (2¼ hours), 2.642 mg (15½ hours) and 8.240 mg (27 hours).

cially its role in agglutinating activity and MPA, both of which are interesting from our point of view. Supposedly, an obligate human pathogen like *B. pertussis* will have an optimum growth temperature of around 37° C and growth at other temperatures would lead to unbalanced growth. To test this supposition, we employed a variety of growth temperatures ranging from 21° C to 42° C. As may be seen from Figure 11, where growth rate (K) is plotted against growth temperature, the doubling time of the culture decreased as the growth temperature increased, with a minimum doubling time at 36–37° C. At this temperature the culture attained its maximum growth rate (K = 0.18 ± 0.026), i.e., the cell population divided every 5.5 hours,
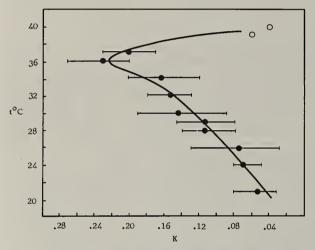
whereas, for example, at 21–23° C the doubling time was trebled. The strain used was highly sensitive to temperatures over 37° C, showing much slower growth at 40° C (20 hours for one doubling). There was no growth at 42° C.

Samples for outer membrane analysis were taken only after the culture had been allowed to stabilize after each temperature adjustment. This was at least 10 times doubling time at lower temperatures and 20 times doubling time at higher temperatures. We found that cells grown at 21° C showed markedly reduced agglutination with factor serum 1 and did not agglutinate at all with factor sera 2 and 3 (Table 5). However, no autoagglutination occurred even after many generations, which indicates that reversion to the R-type did not occur. When stained with diluted carbolfuchsin, the cells appeared as short, slender rods not as strongly stained as those grown at 37° C. When the growth temperature was increased to 26–32° C, the cells readily agglutinated with factor serum 1 and, to a lesser extent, with factor serum 2. However, only at 36–37° C was the full antigenic pattern of the cells developed, i.e., they agglutinated with all three factor sera. This process was fully reversible, indicating phenotypic change as opposed to mutant selection. The agglutinogens always reappeared in the sequence 1–2–3, and were always lost in the reverse order. This means that serotype 1.3 was never achieved by this process.

The amount of crude agglutinogen obtainable from cells of serotypes 1.2 and 1.2.3 was more or less equivalent (6.6–8.5% of dry weight of cells). However, the maximum yield was only 2–3% from cells of serotype 1. When crude agglutinogen obtained from cells of serotype 1.2.3 grown as sero-

Figure 11. Growth constants K determined at different temperatures in semicontinuous cultures of *B. pertussis*, CN 2992. All measurements are based on 10 independent experiments except at temperatures higher than 37° C. K was calculated according to the formula: (log$_2$ dry weight$_2$) − (log$_2$ dry weight$_1$)/time (hrs)

Table 5. Temperature Dependent Changes in Agglutinability and MPA of *B. pertussis* Cells (CN 2992) Grown in Semicontinuous Culture

| Temperature Shift | Age of Culture (hrs.) | No. of Generations | Agglutination Titer with Factor Serum [a] | | | MPA | |
			1	2	3	ED50 μg dry weight	IU per μg dry weight of cells [b]
37°	100	18	512	64	32	1.4	0.028
37→24°C	186	12	4	0	0[c]	47.0	0.000
24→31°C	141	15	16	8	0[c]	38.8	0.004
31→37°C	135	24	512	128	32	1.68	0.023

[a] All agglutinations were performed on the same day using the same batch of factor sera.

[b] Assuming that the total human dose should be contained in a maximum of 1 mg dry weight of the cells, the lowest "specific potency" acceptable for a vaccine is 0.012 IU per μg of dry weight of the cells.

[c] After growth at 24° and 31°C, the culture was inoculated onto agar blood plates and incubated at 37°C. The resulting suspensions showed titers of 512, 64, and 64 with the factor sera 1.2.3 respectively.

type 1 was chromatographed on columns, the usual elution pattern was observed. However, fractions P1 and P11 were unable to inhibit agglutination by factor sera 2 and 3, and in crossed immunoelectrophoresis high concentrations of crude agglutinogen were necessary for detection of the precipitation lines. This shows that the temperature changes induced both quantitative and qualitative differences in the outer membrane composition.

Growth at temperatures other than 36–37° C resulted in cells with a very low level of MPA. When the growth temperature was increased from 26° C to 37° C, the reappearance of agglutinogen 3 coincided with the increase in MPA levels (Table 5), so even the level of MPA appears to be reversible. The time required for the surface layer to recover its full antigenic pattern will be shown in the section describing continuous cultures. However, the reappearance of both the agglutinogens and MPA at 36–37° C was critically dependent on strain history. For example, when a lyophilized or liquid nitrogen-stored culture was used to inoculate agar blood plates, the resultant colonies suspended in defined medium showed agglutination with all three factor sera. However, when this particular suspension was used as an inoculum for the same defined medium in shake flask cultures and in the culture harvested at the end of the exponential growth phase, the agglutination with B. pertussis factor sera was usually weak or, with factor sera 3, absent. The MPA was also greatly reduced. Alternatively, when highly potent frozen suspensions were used directly as inocula for the semicontinuous culture, the lag in the agglutinability of the cells was markedly reduced. This temporary reduction in agglutinability and MPA occurred whenever the medium composition was changed or the fermentation tank was reinoculated.

The transfer of a culture from nutritionally rich to poor medium—"shift down" (35)—leads to severely unbalanced growth, a condition from which B. pertussis recovers slowly. However, when conditions restabilize after several cell divisions, balanced growth may again be attained with the re-expression of the full antigen pattern. These experiments show that B. pertussis can grow for many generations in defined medium. Consequently, we cannot confirm the finding (25) that B. pertussis necessarily loses its protective antigenicity irrevocably after several passages in defined medium. Such loss may be strain-derived or may be due to the presence in the growth medium of inhibitory factors that prevent the stabilization of balanced conditions.

CONTINUOUS CULTURE

For the continuous culture of B. pertussis the fermentor was inoculated with a shake flask culture and growth was allowed to proceed under "batch" conditions until a density of approximately 1.5 mg dry weight of cells per ml was attained. At this density continuous medium addition was commenced at a dilution rate of 0.07–0.09. Conditions with respect to bacterial mass usually stabilized within the next 24 hours. However, preliminary continuous culture experiments with B. pertussis had indicated that the bacterium displayed unusual properties under such conditions. For example, when the dilution rate was increased suddenly in excess of 0.09, or gradually to a rate in excess of 0.13 (critical dilution rate for the medium and strain used, μ_{max}) the cells suddenly died.

This finding was in direct contrast to continuous cultures of other bacteria, where experience has shown that when the dilution rate is increased to above μ_{max} and washout commences, it is still possible to recover the culture by decreasing the medium flow rate and restabilizing the growth conditions at a lower dilution rate. However, such recovery of B. pertussis cultures was impossible; the cells were dead.

Most of our experiments were performed at a dilution rate of 0.1. The cultures were grown at 37° C, pH was kept constant at 7.6 and the impeller speed was adjusted to maintain a 10–20% pO_2 saturation. The cultures were grown under glutamate/proline limitation at concentrations that yielded approximately 1.7 mg dry weight of bacteria per ml (57 μmoles glutamate and 2 μmoles proline per ml). At this density strain CN 2992 gave viable counts of 1–2×10^{10} cfu/ml. At increased glutamate/proline concentrations (in identical molar ratio) higher cell densities were obtained, but maintenance of aerobic conditions became difficult. On the other hand, doubling the cysteine or glutathione ascorbic acid mixture concentrations had no effect on the bacterial mass. Thus these substances were not growth limiting under the conditions used. Complete utilization of glutamate and proline was confirmed by testing the culture supernatants by TLC. The effluent from the tank was formolized either continuously (see Methods) or in bulk twice a day. Formolization lasted 5–24 hours.

During continuous culture the agglutinability

and MPA of the cells were followed (Fig. 12). In this particular example a complete loss of agglutinability by factor sera 2 and 3 occurred at the beginning of the run, but reappeared after 2–7 days. Although such a drastic decrease in the agglutinability of cells was unusual, the example illustrates the subsequent recovery of the cells. The culture displayed significant MPA levels only after the agglutination titer with factor serum 3 had stabilized—a situation similar to that found in semicontinuous cultures. At a 0.1 dilution rate the doubling time of the culture was nearly 7 hours (calculated according to the formula T = 1n2/dilution rate); therefore about 80 generations were necessary to achieve cellular equilibrium.

The speed in which the cells attained their full agglutinability was greatly dependent on the quality of the tank inoculum. Although the agglutination titers of cells always decreased after transfer to a new environment, their recovery was usually faster than in the example shown in Figure 12. Nevertheless, at a dilution rate of 0.1, 8–10 days was usually necessary before the quality of the cells and stabilization of the culture could be assessed.

These experiments confirmed our finding from semicontinuous cultures that *B. pertussis* may be grown in defined medium for many generations. They further showed that bacterial mass alone cannot be considered a reliable criterion for the presence of steady state conditions, because even when bacterial mass remains constant the outer cell membrane may undergo gradual dynamic change until a final equilibrium is reached.
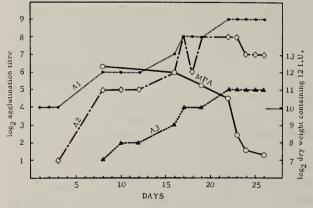
Figure 12. Development of agglutinability and MPA during continuous culture of *B. pertussis* CN 2992 at 37° C. A1, A2, A3: agglutination titer with factor sera 1,2, and 3, respectively. MPA: log₂ of dry weight of cells needed for 12 IU. Short dotted horizontal line represents highest acceptable limit (12 IU/1000 μg cells). Cell concentration was constant at 1.7 mg dry weight of cells per ml.

Criteria of Balanced Growth of *B. pertussis*

The semicontinuous and continuous culture studies showed that the agglutinability of the cells by all three factor sera may be a useful criterion of the complete assembly of the outer membrane. We assumed that cells from a culture showing balanced growth gave maximum agglutination titers. The validity of this assumption is supported by the following additional criteria.

1. *Morphology of cells.* When cells not showing the full agglutinogenic pattern were sectioned, their surface layers appeared folded or crumpled (Fig. 9E), and this appearance was independent of their MPA level. However, the cells displaying the full agglutinogenic pattern were smooth in sections (Fig. 9F) and the cells usually showed high MPA levels. As has been shown by Burdett and Murray (24) with cultures of *E. coli*, during cell division vesicles and sheets of membrane are observed at the site of the cell constriction. The vesicles, which are formed by blebbing of the outer membrane, are released into the surrounding medium as a source of free endotoxin (36). This also occurs in *B. pertussis* (Fig. 9G). These vesicles, which may be sedimented (50,000–100,000 × g) from cell-free culture supernatants, are both morphologically and actively indistinguishable from vesicles obtained by mechanical disintegration of cells and exhibit considerable MPA. They are the source of the MPA known to occur in *B. pertussis* culture supernatants.

Such vesicles resulting from *B. pertussis* cells grown at suboptimal growth temperatures, and which agglutinated only with factor serum 1, had a lipoid appearance (Fig. 9H) and were at least twice as toxic for embryonated eggs (ED₅₀ approximately 100 μg dry weight) as vesicles recovered from balanced cultures.

2. *Optimum formolization time.* As shown in Figure 12, the MPA level of cells from the semicontinuous culture was critically dependent on the time during the growth cycle when the culture was formolized. We call this the formolization time. When formalin was added to samples taken during the exponential growth phase, the cultures were reasonably potent. However, when samples of the same culture were formolized at later stages, the MPA decreased to negligible levels (within 2¼ hours of the end of exponential growth phase the MPA decreased to half of its original level). This critical dependence upon the formolization time was observed only during the initial cycles of semicontinuous culture. After the culture had sta-

bilized, delays in the formolization time had less effect on the final potency. This may be illustrated by comparing cells from a stabilized continuous culture that had been formolized continuously, and cells from the same culture that had been accumulated in an effluent receiver for 24 hours before being formolized in bulk. The respective potencies were 0.044 IU/μg dry weight of cells and 0.029 IU/μg dry weight of cells. Although the formolization time was always important, with optimal results being obtained when formalin addition occurred during the active growth phase, it became less critical after the culture had stabilized.

3. *Stability of MPA in the presence of aluminum hydroxide.* When cells that had been harvested after only a few cycles of semicontinuous culture growth were mixed with aluminum hydroxide (Alhydrogel, 1 mg Al per ml of the final blend), their MPA levels immediately declined (Table 6). None of these "early vaccines" containing Alhydrogel was as potent as corresponding suspensions from which Alhydrogel had been omitted; indeed, in most instances the MPA levels dropped so drastically that the once-potent cell suspensions became useless. (It should be stressed that this feature is not confined to cells harvested from liquid defined medium and may also be observed with cells harvested from the surface of complex media.) However, when Alhydrogel was added to cells from a stabilized culture (i.e., showing the full agglutinogen complement), no decrease in MPA levels was observed (Fig. 13).

4. *Long-term storage life.* The long-term storage life of a formolized culture also depends on the characteristics of the culture at the time of harvesting. Table 7 shows that although the MPA levels

of the unbalanced cultures were satisfactory at the time of blending, in several cases the level decreased by more than 50% within 7–14 months of storage, even at 4° C. None of the suspensions retained its original potency. Alternatively, vaccine prepared from cells displaying the full agglutinogen complement lost no potency when stored at 4° C, and showed only a minimal decrease when stored at 22–28° C (i.e., variable room temperature). In terms of MPA levels the vaccine had a half-life of approximately one year when stored at 37° C (Fig. 13).

Effect of Steel on *B. pertussis*

We have found that *B. pertussis* is extremely sensitive to high quality stainless steel. This effect is particularly marked when the cells are grown in defined medium containing 0.05 M Tris-HCl buffer. The effect may be studied by considering the growth of *B. pertussis* in fermentors of 5, 50, and 100 liter volumes, all of which were made of Krupp's V4A steel. No growth occurred in Stainer-Scholte medium in the 5 liter fermentor, but growth did occur in the 50 liter and 100 liter fermentors, the latter supporting the greater number of generations (Fig. 14A). The ratio of medium volume to steel surface (ml/cm²) was 4, 8, and 9 respectively. The Swedish steel Uddenholm SIS 2343, from which the Biotech fermentors are made, had a similar detrimental effect. For example, their Fl 103 fermentor (a 3 liter vessel consisting of two steel plates abutting a glass cylinder) was filled with 2 liters of the defined medium and inoculated with 300 ml of an actively growing *B. pertussis* culture containing 1.5 mg dry weight of cells per ml. Within 1 hour of inoculation the culture was dead. Here the volume/surface ratio was 11. This particular steel appeared to be the most inhibitory of all the brands tested.

Table 6. Instability of "Unbalanced" *B. pertussis* Vaccine Blends due to Alhydrogel Addition

Experimental Vaccine Blend [a]	MPA IU per ml [b]	
	Without Alhydrogel	With Alhydrogel
B1– 75	10.68	1.60
B1–100	12.18	3.86
B2– 75	10.43	6.00
B2–100	8.35	8.12
B6	16.0	8.18
B9	9.00	2.80

[a] The vaccine blends contained per ml:
525–630 μg dry weight of bacterial cells
65 Lf diphtheria
12.5 Lf tetanus toxoid and
1 mg Al (as Alhydrogel) when appropriate.
[b] The potencies are the arithmetical mean of four independent potency tests.

Table 7. Instability of *B. pertussis* Vaccine Blends from "Unbalanced" Semicontinuous Cultures

Experimental Vaccine Blend [a]	MPA IU per ml [b]	
	At time of blending	After storage for 7-14 months at 4°C
6A	10.88	7.85
6C	14.45	5.00
6N	18.12	16.00
9A	23.85	13.50
9C	8.48	5.23
9N	9.56	6.70

[a] Similar composition as in Table 6 (without Alhydrogel).
[b] MPA—arithmetical mean of four independent tests.
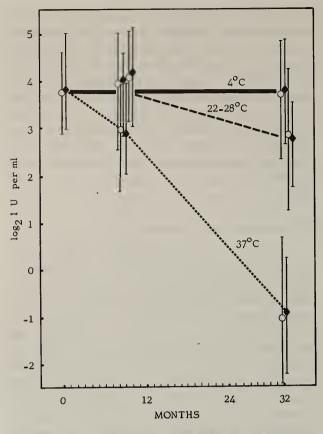
Figure 13. Stability at different temperatures of *B. pertussis* vaccine blends containing cells from "balanced cultures." Blends contained: *B. pertussis* (650 μg/ml), diphtheria and tetanus toxoids (65 and 12.5 Lf/ml respectively) and Alhydrogel (1 mg Al/ml). Bars represent 95% fiducial limits.
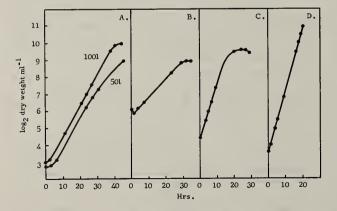
Figure 14. Growth of *B. pertussis*, 366TS, in various fermentors: (A) Stainless steel fermentor, Krupp's V4A steel, defined medium 50 and 100 liters. (B) Stainless steel-glass fermentor, steel EN56B, defined medium 3 liters. (C) As in (B) but with casein-hydrolysate medium. (D) All glass fermentor, defined medium, 70 liters.

On the other hand, lower quality steels that are slightly magnetic, such as the EN56B (supplied by R. G. Brown Stainless-Steel Ltd., London N16), did support moderate growth in defined medium when used in vessels of similar design to the Fl 103 fermentor (Fig. 14B). It appears that the additives used in the manufacture of high quality stainless steels are responsible for the inhibitory effect. It is known, for instance, that manganese is inhibitory to small inocula of *B. pertussis* (37). Although the steel effect is less pronounced when complex media are used (Fig.14C), we were never able to achieve totally balanced cells (i.e., with a smooth surface) in any fermentor made from steel, although the MPA values obtained appeared satisfactory. Therefore, to achieve balanced growth of *B. pertussis* we avoided all contact with stainless steel during both medium preparation and culture growth.

Growth in Casein Hydrolysate Medium

The gradual buildup of the surface layers of *B. pertussis* also occurs when the cells are grown in casein hydrolysate medium, but usually fewer generations are necessary for physiological recovery when noninhibitory casein hydrolysate is used (1,3). As may be seen from Table 8, this also occurs when *B. pertussis* is grown on solid casein hydrolysate medium. Two cultures of *B. pertussis* grown in liquid medium and containing widely differing MPA and agglutinogen levels were used to inoculate two sets of 50 Roux flasks, each containing 500 ml solid casein hydrolysate medium. The flasks were incubated at 37° C and sampled daily for 3 days, each sample being resuspended in PBS, formolized (0.25% formalin) and tested for relative potency. The table shows that the significant difference in MPA levels between the two sets of cultures after 24 hours was greatly reduced after 72 hours incubation.

DISCUSSION

According to the formal definition of Maaløe and Kjeldgaard (38), balanced growth is "the result of a definite partitioning of energy and matter among all the components being synthesized"—a state of total harmony of regulation between the cytoplasm, cell wall, and membranes of the bacterial cell during growth. This is in contrast to unbalanced growth, where no such harmony exists and the synthesis of certain specific macromolecules and structures is favored (33,35,38–40). Extremely unbalanced growth leads to osmotic fragility of

Table 8. Recovery of MPA of *B. pertussis*, CN 2992, During Growth on Solid Casein-Hydrolysate Medium

Inoculum	MPA IU/single dose (95% limits) after incubation		
	24 hours	48 hours	72 hours
Series A. Culture with low MPA and agglutinogen levels	1.88 (0.89–4.00)	3.09 (1.77–5.40)	6.13 (3.55–10.59)
Series B. Culture with high MPA and agglutinogen levels	9.63 (5.68–16.33)	6.21 (3.65–10.56)	7.18 (4.29–12.00)

cells, to thickening of the cell wall, and/or to defective cell divisions with resultant death of the cell (39,40). However, due to the great adaptability of bacteria, the less pronounced forms of unbalanced growth may be manifested as an endless number of relatively stable phenotypes able to multiply in steady state (i.e., at a constant increase of bacterial mass per time unit). From this it follows that recognition of the balanced state is easier in well-studied species or strains, such as *Streptococcus faecalis* 9790 in Shockman's studies (39), but relatively difficult in bacteria whose physiology is not yet well understood, and where criteria for the identification of a harmonious state do not exist.

Our criteria for the balanced state in *B. pertussis* are provisional and greatly influenced by the needs of the vaccine producer to obtain cells with the necessary antigenic pattern that remain both stable and relatively nontoxic. We do not know whether these characteristics reflect a harmonious state with respect to the cell itself. However, the morphological appearance of the cells during growth and their stability under suboptimal storage conditions seem to reflect the degree of balance between the intrinsic autolytic system and a steady buildup of the membranes. To obtain a deeper understanding of pathogens such as *B. pertussis*, their growth should also be studied during the course of the natural infection. Nevertheless, we assume for the present that the simplest criterion for the identification of the balanced state in *B. pertussis* cells is agglutinogen content. We consider that *cells that for several generations have shown maximum agglutination levels with factor sera 1 (2) and particularly 3 have completely formed surface layers* and are smooth in appearance in thin sections and relatively stable during harvesting and formolization, and can maintain high stability during storage. Such cells also showed low toxicity in weight gain tests and acceptable MPA levels, although MPA alone, irrespective of titer, does not reflect balanced conditions; unbalanced cells may show a

high MPA level that usually declines at an unpredictable rate during storage. Cells from cultures that would be described as balanced according to the above criteria presented no problems in the mouse-protection test. However, with cells originating from unbalanced cultures, the test or the level of the testing very often had to be repeated to obtain acceptable 95% limits.

We believe that the relationship between agglutinogens, particularly agglutinogen 3, and the protectivity of a vaccine is indirect. A direct relationship, as proposed by Preston and Stanbridge (41,43), appears unlikely for two reasons. First, in passive protection tests the agglutinogen content of serum does not correlate with its protective power (10). Second, we have recently found strains of *B. bronchiseptica* that agglutinate with *B. pertussis* factor sera 1, 2, and 3, but are unable to protect against *B. pertussis* 18323 challenge in the MPT (Cownley and Novotny, unpublished). Nevertheless, the epidemiologic data presented by Preston and his associates (41,42,44) are very valuable and are in good agreement, albeit circumstantially, with our study of the physiology of *B. pertussis*. When analyzing his epidemiological data, Preston concluded that factor 3 is important in vaccines. We came to the same conclusion, since the surface layers of cells with a high factor 3 content appear to have a more complete and stable structure and may induce better protection in children. However, since we believe that agglutinogen content and protective activity are independent characters, we do not share the view (44) that the MPT is irrelevant.

The variability in pattern of agglutinogens 1, 2, and 3 in *B. pertussis* is well known. This has recently been studied from several aspects (45–47), and it appears that their formation may be suppressed by various inhibitors in the culture medium. However, Preston and Stanbridge (44) have recently found that a very well known and notorious factor-1 strain 353/Z (48,49) may also display factor 2 or 3 in vivo when inoculated into the naso-

pharynx of marmosets. It appears therefore that *B. pertussis* strains differ primarily in sensitivity to agglutinogen suppression and that previously recorded serotypes may in fact be derivatives of either serotype 1.2.3 or 1.3.

Using a solid medium, Lacey (50) showed that the total or partial replacement of NaCl by various salts, particularly Mg⁺⁺, induced phenotypic changes to the surface of *B. pertussis* cells, an effect he has termed modulation. He observed that such changes, whether induced by medium composition or by growth temperature reduction (25° C), resulted in the manifestation of an infinite number of predictable antigenic states, with the transition from one such state to another requiring 7–15 cell divisions. He suspected that these changes stemmed from altered rates of synthesis of at least three kinds of antigen molecules, a good example of unbalanced growth according to the above criteria.

In our experiments, where the medium composition remained constant, the changes in phenotype, whether temperature-induced or spontaneous (i.e., at the beginning of growth), seem to depend on the temperature optima of the enzymes involved and on the regulatory instability of *B. pertussis*. This is most apparent in continuous cultures where, after a sudden change in flow rate, the rapid death of the cells is reminiscent of substrate-accelerated death. This phenomenon, which in *Klebsiella aerogenes* may be prevented by the addition of cyclic AMP (51), is therefore most probably related to intracellular repression and depression mechanisms. The study of the formation and secretion of the recently discovered unique adenylate cyclase of *B. pertussis* (52) may elucidate this regulatory instability.

Nowadays, membranes from Gram-negative bacteria are usually prepared from osmotically shocked lysozyme-EDTA spheroplasts, with subsequent separation in sucrose gradient (26). Although the preparations so obtained are relatively pure the procedure is laborious. The use of the rotary disintegrator (4) followed by differential centrifugation seems especially suitable for the isolation of the outer membrane of *B. pertussis*. Although no enzymatic studies of such preparations have yet been undertaken, the vesicles obtained showed a high homogeneity under the electron microscope and in Metrizamide gradient. After phenol treatment they were transformed into LPS fibers, which in *B. pertussis* may be used as a suitable criterion of their identity. The rotary disintegrator's effectiveness appears to lie in its gentle action. Due to its fluidlike character, the outer membrane is stripped off sooner than, for instance, pili in *S. typhimurium* (Fig. 9I). However, the final preparation obtained from *S. typhimurium* was not as homogeneous as that obtained from *B. pertussis;* therefore its effect cannot be generalized. On the other hand, using the French press, Mickle homogenizer, or MSE 150 Watt Ultrasonic Distintegrator, we were unable to obtain a similarly homogeneous preparation from *B. pertussis*. Further fractionation of the membranes by sucrose gradient to separate the inner and outer membrane fractions (53) is necessary with these processes.

Since the scaling-up and commercial availability of an industrial "rotary disintegrator" (54), large-scale operation should be no problem. However, all of the procedures so far used to fractionate *B. pertussis* into its protective fraction, whether mechanical (6, 55–60) or chemical in principle, e.g., by SDS treatment (61,62), have much in common. They separate, with varying effectiveness, the outer membrane fraction, which may then be sedimented at 50,000–100,000 × g and represents the natural endotoxin of *B. pertussis*. One of these preparations in the form of Pillemer's SPA has already been tested in the field trial, and has shown high protective activity coupled, unfortunately, to a higher level of toxicity in children (17) that discouraged its further use. One may assume therefore that other preparations of this type would behave similarly. Further trials appear to be unethical unless the lipopolysaccharide moiety is removed. This appears to be a most difficult task.

The components of the outer membrane of *B. pertussis* so far identified are schematically represented in Figure 15. We suppose that the backbone of the outer membrane consists of lipopolysaccharide in a latticelike arrangement (Fig. 9D). This is based on the observation that the LPS content of the cells remained more or less stable, whereas the other components varied according to the cultural conditions. The macromolecular structure of the LPS, which appears to be finer than that seen in gonococcus (31), is damaged by phenol treatment, being transformed into fibers. A similar fibrillar LPS has also been reported by Nakase et al. (63). The outer membrane proteins may be separated by mild acid hydrolysis, a method which in another form was useful in the separation of membrane proteins from *S. typhimurium* (64) and also in the separation of agglutinogens from *B.*
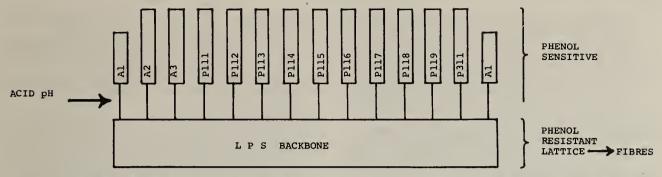
Figure 15. Schematic representation of the outer membrane of *B. pertussis*. LPS—Lipopolysaccharide. A1, A2, A3—agglutinogens (haptens?). P111–P119, P311—outer membrane proteins identified by crossed immunoelectrophoresis. P311 is genus-specific (except for *B. bronchiseptica*, serotype K 1.3.4; O 4). P112–P114 are common to *B. pertussis* and *B. parapertussis*. The relationship of the outer membrane proteins to labile deoxysugars (deoxysugar acid and pertussose) is unknown. P311 probably corresponds to Ag7 of Hertz et al. (27).

pertussis (65). Outer membrane proteins are usually characterized in SDS-acrylamide gels (26,53,64).

Because of the low reactivity of the small proteins obtained from *B. pertussis* with Coomassie Brilliant Blue, these methods were not very useful unless combined with immunologic detection of antigens (unpublished observation). The antigenic diversity of these proteins became apparent, however, in crossed immunoelectrophoresis; at least 10 different antigens were identified (P111–P119, P311). It appears that the proteins are connected to the LPS backbone by weak covalent bonds.

During the hydrolysis, the liberation of labile sugars was always observed. These sugars react in the 2-thiobarbituric acid assay. One is acidic in character, while the other, which we have termed pertussose, is neutral. However, neither of them behaves like typical KDO. Although these sugar components are always liberated, their relationship to the rest of the subunits is not known. The surprising thing about the protein components of the outer membrane was that their molecular weights were very similar—around 10 million—which makes it impossible to separate them on Sephadex G–25. Most of them had very similar electrophoretic mobilities in agarose gel at pH 8.6, except P119 and P311. The only free amino-end that we could find was the ε-amino-group of lysine. The agglutinogens, which were also removed from the outer membrane by mild acid treatment, did not precipitate with factor sera 1, 2, or 3, but were able to inhibit the agglutination of whole cells by these sera. They are probably haptens. Although agglutinogens 2 and 3 were eluted from Sephadex G–25 together with the majority of the outer membrane proteins, agglutinogen 1 was retarded in the column under similar conditions and its molecular weight appears to be

smaller. Fragmentation of the outer membrane by the various techniques so far used ultimately leads to the disappearance of the mouse-protective activity.

REFERENCES

1. Pekárek, J.; Stejskal, A. Preparation of non-reactive and non-sensitizing vaccine. Round Table Conference on Pertussis Immunization, Prague; 1962.
2. Stainer, D. W.; Scholte, M. J. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. J. Gen. Microbiol. 63:211–220; 1970.
3. Stejskal, A.; Stránsky, V.; Stejskal, V. 1966. Procéé de fabrication du produit intermédiaire pour le vaccine pertussis-parapertussis. Czechoslovak Patent No PV3.397/66. Basic Prov. F4386 USTS 20.5. 66 35810.
4. Novotny, P. A simple rotary disintegrator for microorganisms and animal tissue. Nature 202:364–366; 1964.
5. Salton, M. R. J.; Horne, R. W. Studies of the bacterial cell wall. II. Methods of preparation and some properties of cell walls. Biochim. Biophys. Acta 7:177–197; 1951.
6. Pillemer, L.; Blum, L.; Lepow, I. H. Protective antigen of *Haemophilus pertussis*. Lancet 1:1257–1260; 1954.
7. Novotny, P.; Cownley, K.; Smith, F. A. L-layer of cell walls of *Bordetella pertussis*: its relation to endotoxin, serological and protective properties. Proc. Soc. Gen. Microbiol. 3:29–31; 1975.

8. Novotny, P.; Cownley, K. Free and phenol endotoxin of *B. pertussis*. Proc. Soc. Gen. Microbiol. 4:33; 1976.

9. Public Health Lab Service Whooping-Cough Committee & Working Party. Efficacy of whooping-cough vaccines used in the U.K. before 1968. Br Med J 1:259–262; 1973.

10. Novotny, P.; Brookes, J. E. The use of *Bordetella pertussis* preserved in liquid nitrogen as a challenge suspension in the Kendrick mouse protection test. J. Biol. Stand. 3:11–29; 1975.

11. Novotny, J.; Broughton, E. S.; Cownley, K.; Hughes, M.; Turner, W. H. Strain related infectivity of *Neisseria gonorrhoeae* for the guinea-pig subcutaneous chamber and the variability of the immune resistance in different breeds of guinea-pigs. Br. J. Vener. Dis. 54:88–96; 1978.

12. Birine, G. D.; Rickwood, D.; Hell, A. Buoyant densities and hydration of nucleic acids, proteins and nucleo-protein complexes in metrizamide. Biochim. Biophys. Acta 331:283–294; 1973.

13. Elbein, A. D.; Heath, E. C. The biosynthesis of cell wall lipopolysaccharide in *Escherichia coli*. J. Biol. Chem. 240:1919–1925; 1965.

14. Ashwell, G. New colorimetric methods for sugar analysis. Methods Enzymol. 8:85–95; 1966.

15. Westphal, O.; Lüderitz, O.; Bister, F. Über die Extraktion von Bakterien mit Phenol/Wasser. Z. Naturforsch. 7b:148–155; 1952.

16. Kabat, E. A.; Mayer, M. M. Kjeldahl nitrogen determination. Modification for samples of 10–100 micrograms. Kabat and Mayer's experimental immunochemistry, 2nd edition. Springfield, Illinois: Charles C. Thomas; 1961: 480–482.

17. Medical Research Council. Vaccination against whooping-cough. Br. Med. J. 1:994–1000; 1959.

18. Hawort, C.; Heathcote, J. G. An improved technique for the analysis of amino acids and related compounds on thin layers for cellulose. J. Chromatogr. 41:380–385; 1969.

19. Gray, W. R. End-group analysis using dansyl chloride. Methods Enzymol. 25B:121–138; 1972.

20. Warren, L. Thiobarbituric acid spray reagent for deoxy sugars and sialic acids. Nature 186:237; 1960.

21. Andersen, E. K. Serological studies on *H. pertussis, H. parapertussis* and *H. bronchisepticus*. Acta Pathol. Microbiol. Scand. B. 33:202–224; 1953.

22. Smyth, C. J.; Siegel, J.; Salton, M. R. J.; Owen, P. Immunochemical analysis of inner and outer membranes of *Escherichia coli* by crossed immunoelectrophoresis. J. Bacteriol. 133:206–319; 1978.

23. Csizér, Z.; Szammet, Á.; Niedermayer, N.; Zsidai, J.; Aubert, E.; Joó, I. Estimation of bacterial mass of pertussis vaccine by opacity and dry weight determinations. J. Biol. Stand. 5:289–295; 1977.

24. Burdett, I. D. J.; Murray, R. G. E. Septum formation in *Escherichia coli:* Characterization of septal structure and the effects of antibiotics on cell division. J. Bacteriol. 119:303–324; 1974.

25. Parker, C. Role of the genetics and physiology of *Bordetella pertussis* in the production of vaccine and study of host-parasite relationship in pertussis. Adv. Appl. Microbiol. 20:27–42; 1976.

26. Osborn, M. J.; Gander, J. E.; Parisi, E.; Carson, J. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of cytoplasmic and outer membrane. J. Biol. Chem. 247:3962–3972; 1972.

27. Hertz, J. B.; Høiby, N.; Andersen, V.; Baekgaard, P. Crossed immunoelectrophoretic analysis of *B. pertussis* antigens and of corresponding antibodies in human sera. Acta Pathol. Microbiol. Scand, B. 84:386–394; 1976.

28. Westphal, O.; Lüderitz, O. 3,6-Didesoxy-hexosen-chemie und biologie. Angew. Chem. 72:881–891; 1960.

29. Chaby, R.; Szabo, L. 3-Deoxy-2-octulosonic acid 5-phosphate: a component of the endotoxin of *Bordetella pertussis*. Eur. J. Biochem. 59:277–280; 1975.

30. Sato, Y.; Nagase, K. Isolation of protective antigen from *Bordetella pertussis*. Biochem. Biophys. Res. Commun. 27:195–201; 1967.

31. Novotny, P.; Cownley, K. Immunological anatomy of *Neisseria gonorrhoeae*. Brooks, G. F.; Gotschlich, E. C.; Holmes, K. K.; Sawyer, W. D.; Young, F. E. eds. Immunobiology of *Neisseria gonorrhoeae*. Washington, D.C.: American Society for Microbiology; 1978; 263–271.

32. Csizer, Z.; Joó, I. Large-scale production of pertussis vaccine by serial subculture technique. Zentralbl. Bacteriol. (Orig. A) 221:59–64; 1972.

33. Kjeldgaard, N. O.; Maalóe, O.; Schaechter, M. The transition between different physiological states during balanced growth of *Salmonella typhimurium*. J. Gen. Microbiol. 19:607–616; 1958.

34. Fencl, Z. Homogeneous single-stage semicontinuous and continuous cultivation. Malek, I.; Fencl, Z., eds. Theoretical and methodological basis of continuous culture of microorganisms. New York: Academic Press, Inc.; 1966: 79–88.

35. Schaechter, M.; Maaløe, O.; Kjeldgaard, N. O. Dependency on medium and temperature of cell size and chemical composition during balanced growth of *Salmonella typhimurium*. J. Gen. Microbiol. 19:592–606; 1958.

36. Crutchley, M. J.; Marsh, D. G.; Cameron, J. Free endotoxin. Nature 214:1052; 1967.

37. Cameron, J.; Dunning, J. M. Inhibition of the growth of *Bordetella pertussis* from small inocula by manganese in agar. *In* New vaccine of gram-negative bacili. International Congress for Microbiology, Mexico City: Government of Mexico; 1970.

38. Maaløe, O.; Kjeldgaard, N. O. Control of macromolecular synthesis. New York–Amsterdam: W. A. Benjamin, Inc.; 1966.

39. Shockman, G. D. IV. Unbalanced cell wall synthesis: autolysis and cell wall thickening. Bacteriol. Rev. 29: 345–358; 1965.

40. Shockman, G. D.; Daneo-Moore, L.; Higgins, M. L. Problems of cell wall and membrane growth, enlargement and division. Ann. NY Acad. Sci. 235:161–197; 1974.

41. Preston, N. W.; Stanbridge, T. N. Efficacy of pertussis vaccines: a brighter horizon. Br. Med. J. 3:448–451; 1972.

42. Preston, N. W. Prevalent serotypes of *Bordetella pertussis* in non-vaccinated communities. J. Hyg. (Camb.) 77:85–91; 1976.

43. Stanbridge, T. N.; Preston, N. W. Experimental pertussis infection in the marmoset: type specificity of active immunity. J. Hyg. (Camb.) 72:213–228; 1974.

44. Preston, N.W.; Stanbridge, T. N. Mouse or man? Which are pertussis vaccines to protect? J. Hyg. (Camb.) 76:249–256; 1976.

45. Holt, L. B.; Spasojević, V. The role of surface antigens in the protective potency of *Bordetella pertussis* suspensions as measured by the intracerebral challenge technique in mice. J. Med. Microbiol. 1:119–126; 1968.

46. Cameron, J. Variation in *Bordetella pertussis*. J. Pathol. Bacteriol. 94:367–374; 1967.

47. Bronne-Shanbury, C. J.; Dolby, J. M. The stability of the serotypes of *Bordetella pertussis* with particular reference to serotype 1,2,3,4. J. Hyg. (Camb.) 76:227–286; 1976.

48. Eldering, G.; Holwerda, J.; Baker, J. *Bordetella pertussis* culture having only species factor 1. J. Bacteriol. 91:1759–1762; 1966.

49. Ross, R. F.; Munoz, J. Antigens of *Bordetella pertussis*

V. Separation of agglutinogen 1 and mouse-protective antigen. Infect. Immun. 3:243–248; 1971.

50. Lacey, B. W. Antigenic modulation of *Bordetella pertussis*. J. Hyg. (Camb.) 58:57–93; 1960.

51. Calcott, P. H.; Postgate, J. R. On substrate-accelerated death in *Klebsiella aerogens*. J. Gen. Microbiol. 70: 115–122; 1972.

52. Hewlett, E. L.; Urban, M. A.; Manclark, C. R.; Wolff, J. Extracytoplasmic adenylate cyclase of *Bordetella pertussis*. Proc. Nal. Acad. Sci. USA 73:1926–1930; 1976.

53. Schnaitman, C. A. Protein composition of the cell wall and cytoplasmic membrane of *Escherichia coli*. J. Bacteriol. 104:890–901; 1970.

54. Rehacek, J.; Schaefer, J. Disintegration of microorganisms in an industrial horizontal mill of novel design. Biotechnol. Bioeng. 19: 1523–1534; 1977.

55. Billaudelle, H.; Edebo, L.; Hammarsten, E.; Heden, G-C.; Malmgren, B.; Palmstierna, H. Studies on the chemical and immunological structure of *Bordetella pertussis*. Acta Pathol. Microbiol. Scand. B 50:208–224; 1960.

56. Munoz, J.; Hestekin, B. M. Antigens of *Bordetella pertussis*. lll. The protective antigen. Proc. Soc. Exp. Biol. Med. 112:799–805; 1963.

57. Schuchard, L. F.; Munoz, J.; Verwey, W. F.; Sagin, J. F. The relationship of agglutinogen to other antigens of *Bordetella pertussis*. J. Immunol. 91:107–111; 1963.

58. Sato, Y.; Nagase, K. Studies on protective antigen of *Bordetella pertussis*: isolation of protective antigen by sucrose density gradient centrifugation and some properties of the antigen. Jpn. J. Med. Sci. Biol. 18:173–174; 1965.

59. Nakase, Y.; Fujita, H.; Takatsu, K.; Yoshioka, M.; Kasuga, T. lmmunochemical study on antigenic compounds of *Bordetella pertussis*. Jpn. J. Med. Sci. Biol. 18:170–171; 1965.

60. Mucke, W.; Mebel, S.; Zacharova, M. S.; Lapaeva, I. A. Zur serologie von *Bordetella pertussis*. 11. Herstellung von Ultraschallextrakten aus *B. pertussis* und ihre Weiterverarbeitung an DEAE-zellulose Säulen. Zentralbl. Bacteriol. (Orig A) 221:65–69; 1972.

61. Nagel, J. lsolation from *Bordetella pertussis* of protective antigen free from toxic activity and histamine sensitizing factor. Nature 214: 96–97; 1967.

62. Barta, G. Soluble protective antigen from *Bordetella pertussis* prepared with sodium desoxycholate. J. lmmunol. 90:72–80; 1963.

63. Nakase, Y.; Tateisi, M.; Sekiya, K.; Kasuga, T. Chemical and biological properties of the purified O antigen of *Bordetella pertussis*. Jpn. J. Microbiol. 14:1–8; 1970.

64. Schnaitman, C. A. Examination of the protein composition of the cell envelope of *Escherichia coli* by polyacrylamide gel electrophoresis. J. Bacteriol. 105:882–889; 1970.

65. Onoue, K.; Kitagawa, M.; Yamamura, Y. Chemical studies on cellular components of *B. pertussis*. l. Purification and properties of agglutinogen. J. Bacteriol. 82:648–656; 1961.

Differences Observed Between Fresh Isolates of *Bordetella pertussis* and Their Laboratory Passaged Derivatives

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ABSTRACT

Fresh isolates of *B. pertussis* were passaged on Tryptic Soy Agar (TSA) containing decreasing amounts of blood in order to obtain derivatives that would grow on TSA. Three of the derivative strains that were isolated were partially characterized and were shown to vary in serological and biological properties. Only one of the three, UT 21(32), showed a uniform loss of properties when compared with its parental strain, UT 21(5). Strain UT 21(32) failed to agglutinate with three different antisera, and no longer produced dermonecrotic toxin. A vaccine prepared from strain UT 21(32) afforded little protection to mice and failed to sensitize mice to the lethal effects of histamine or produce elevated white counts at the dosage given.

Strains UT 21(5) and UT 21(32) were further tested for susceptibility to antibiotics and for sensitivity to saturated and unsaturated fatty acids. Strain UT 21(32) exhibited increased resistance to penicillin and erythromycin when compared with strain UT 21(5). Strain UT 21(32) was more resistant to saturated fatty acids containing 10–16 carbons and to most unsaturated fatty acids tested.

INTRODUCTION

Bordetella pertussis is a fastidious, slow-growing bacterium that is difficult to isolate on laboratory media. Once isolated and subcultured several times, it is known to undergo rapid in vitro variation. The antigenic changes that occur with repeated subculture were characterized by Leslie and Gardner in 1931 (1). They used the designations phases I, II, III, and IV to describe the progressive loss of antigens by fresh isolates during subculture, resulting in antigenic variants. Standfast (2), in 1951, described the changes that occur in *B. pertussis* strains after repeated subculture on Bordet-Gengou medium. He found that growth requirements, agglutination, virulence, protective antigenicity, hemagglutination, alum precipitation, bile solubility, and morphology varied independently.

More recently, Parker has suggested the terms fresh isolate, intermediate strain, and degraded strain be used to include all the changes that occur during in vitro passage of pertussis strains (3). She hypothesized that changes brought about by serial culture of pertussis are selected for by inhibitors present in various culture media.

The object of our research was to serially pass fresh isolates of *B. pertussis* to obtain derivative strains that would grow on ordinary nutrient medium without blood, and to determine how such derivative strains differ from their parental strains. We describe here a systematic method for obtaining derivative strains from fresh isolates of *B. pertussis* and present the preliminary characterization of three such pairs.

MATERIALS AND METHODS

Glassware

All glassware, including pipettes, was acid-cleaned, and rinsed extensively in distilled, deionized water.

Organisms

B. pertussis strains UT 21, UT 25, and UT 26 were isolated in our laboratory during an outbreak of pertussis in 1975 (4). Each strain was isolated on Bordet-Gengou (BG) medium without peptone (4) and was transferred once on this medium before being frozen in blood or lyophilized as part of our culture collection [UT 21(1), UT 25(1), and UT 26(1)]. (In this paper, the number in parentheses following the strain number refers to the number of subcultures from original isolation.)

To obtain derivatives that would grow on medium without blood, each fresh isolate was passaged serially on Tryptic Soy Agar (TSA) (Difco Labora-

tories) containing decreasing amounts of defibrinated sheep blood. The procedure used to obtain these derivatives is described in detail below and presented diagrammatically in Figure 1.

Initially, each isolate was tested for ability to grow on TSA as follows. A heavy inoculum of each strain was transferred to a fresh BG plate and to a TSA plate containing 5%, 2%, 1% and 0% blood. Plates were incubated for 5 days at 35° C in a moist chamber, and growth was then scored using an arbitrary scale of 4+ to 0. On this scale, 4+ represented growth equivalent to that seen on the BG plate, and 3+, 2+, and 1+ represented 75%, 50%, and 25%, respectively, of the growth seen on BG; 0 represented no growth.

At this point, each strain [UT XX(2)] displayed the same pattern of growth 4+ growth on TSA + 5% blood; 4+ growth on TSA + 2% blood; 2+ growth on TSA + 1% blood; and no growth on TSA. To determine whether growth had been initiated as the result of a carryover of nutrients from the original BG plate, each strain was transferred again onto the same medium and incubated as above. At the end of 5 days, each strain again showed the same pattern of growth, except that no growth was seen on TSA + 1% blood.

Since growth on TSA +5% blood and TSA + 2% blood seemed equivalent, growth from the 2% blood plates was used for further serial passages to try to obtain a derivative of each strain that would grow on TSA without blood. Thus each strain was further passaged on both TSA + 2% blood and TSA + 1% blood until growth on TSA + 1% blood was obtained. In a like manner, passage of each strain on TSA + 1% blood was continued, and growth from these plates was used to heavily inoculate sectors of TSA plates. After consistent growth on sectors of TSA was obtained, the growth from each sector was used to heavily inoculate entire plates of TSA. After a variable number of passages on TSA to insure stable growth, each strain was lyophilized for use in these studies.

Further passage of these strains on TSA is continuing in our laboratory. Various intermediate stages of each strain have also been preserved but have not yet been studied. In this report, we define fresh isolates, intermediate strains, and derivative strains of B. pertussis as follows. A *fresh isolate* is a strain that has been passed a minimum number of times since isolation and maintained only on BG medium without peptone. An *intermediate strain* is one that is able to grow on TSA with small amounts of blood, but not on TSA alone. A *derivative* strain is one that exhibits stable growth on TSA medium without blood.

Growth of Fresh Isolates and Derivative Strains in Liquid Media

In order to investigate differences between each fresh isolate and its derivative strain, each was grown in Stainer-Scholte (SS) medium, modified to contain 1.52 g/l of Trizma Base (5). Frozen seed cultures, used to inoculate liquid cultures of SS medium, were prepared for each strain as follows. Each strain was cultured from a lyophile vial onto the appropriate medium and incubated for 72 hours at 35° C. Fresh isolates were grown on BG medium and derivative strains on TSA. Each strain was then subcultured onto the same medium and incubated for an additional 48–72 hours. Growth from these plates was harvested into SS medium containing 10% glycerol. Each suspension was adjusted to 175–185 Klett units (KU) using a Klett-Summerson colorimeter at 540 nm. Seed cultures were divided into 5 ml samples, quick-frozen in a dry ice/ethanol bath and stored at −70° C. Vol-
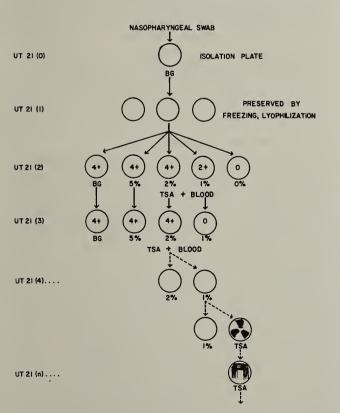

Figure 1. Method for obtaining derivative strains of B. pertussis. Representative subcultures are shown on the left for B. pertussis strain UT 21.

umes of 1–5 ml of frozen seed culture were used to inoculate liquid cultures consisting of 50 ml SS medium in 500 ml nepheloflasks. Liquid cultures were incubated for 18–24 hours at 35° C in a rotary shaker (180 rev/min) until late exponential growth was reached (150–175 KU). These cultures were used for preparation of whole cell vaccines, for serological testing, and as inocula for growth studies with various fatty acids and antibiotics, as described below.

Preparation of Whole Cell Vaccines

Each fresh isolate and its derivative strain were grown in SS medium until late exponential growth phase was reached. Cells were harvested by centrifugation, resuspended in sterile, nonpyrogenic saline, and adjusted to 175 KU using the Klett-Summerson colorimeter (540 nm). A portion of each vaccine was saved to test for the presence of dermonecrotic toxin (DNT). The remainder was heated to 56° C for 30 minutes. Merthiolate was added to a final concentration of 1:10,000, and the vaccines were stored at 4° C until tested for biological activities.

Serological Tests

Antisera. Antisera against *B. pertussis* were prepared in two rabbits as previously described (4). Antiserum from rabbit A was used both for tube agglutination tests and for preparation of a fluorescent antibody conjugate. The U.S. Standard Antipertussis Serum (Rabbit conc; Lot No. 2) was kindly provided by Dr. Charles R. Manclark, Bureau of Biologics, Bethesda, Maryland.

Agglutination tests. Tube agglutination tests were carried out using a modification of the procedure described by Eldering, Eveland, and Kendrick (6). Dilutions of the three separate antisera (rabbit A, rabbit B, and Lot No. 2) were prepared in saline, and 0.1 ml amounts were placed in 10 × 75 mm test tubes. A volume of 0.1 ml of a live antigen suspension (adjusted to 150 KU) was added to each tube. Tubes were shaken for 2 minutes by hand, and then were incubated for 1 hour in a 37° C water bath. The tubes were allowed to stand overnight at 4° C before being read for agglutination. The titer for each antiserum was read as the reciprocal of the last tube showing a 2+ agglutination reaction.

Fluorescent antibody (FA) staining. An FA conjugate was prepared from antiserum A according to the procedure of Hebert et al. (7). The conjugate had a final fluorescein/protein ratio of 25 μg of bound fluorescein isothiocyanate per mg of protein per ml of conjugate. The procedure for direct FA staining was carried out as outlined by Pittman (8). Briefly, twofold dilutions of the conjugate (from 1:2–1:2048) were prepared in 0.01 M phosphate buffered saline (PBS), pH 8.0. Smears of organisms were prepared from a suspension of cells adjusted to 150 KU. Smears were heat-fixed and stained for 30 minutes with each dilution of the conjugate. Slides were rinsed for 10 minutes in PBS, allowed to air dry, and mounted using buffered glycerol-saline, pH 9.0. Slides were read with a Zeiss fluorescence microscope using an HBO 200 mercury arc lamp and incident light illumination. The brightness and relative contrast of each smear was judged visually using a scale of 4+ to 0. The titer was defined as the reciprocal of the highest dilution of conjugate that gave complete (2+) staining of the organisms.

Factor typing. Factor typing was kindly performed by Dr. Dennis Stainer, Connaught Laboratories Limited, Ontario, Canada. Lyophilized cultures sent to him were opened onto BG plates and were relyophilized after growth. The lyophiles were cultured onto BG plates and grown for 4 days at 37° C. Bacterial lawns scraped from the plates were suspended in saline and mixed with specific antisera against factors 1,2, and 3. The agglutination pattern was judged with the following scale: ++, fast (1 to 2 min); +, slow (3 to 5 min); ±, very slow (6 min); and −, no agglutination.

Tests for Biological Activity

Dermonecrotic toxin (DNT). DNT was measured as previously described (9) using 4-day-old CFW mice. Fivefold dilutions of organisms were prepared from 24-hour liquid cultures adjusted to 150 KU. Two mice were injected for each dilution tested. After 18–24 hours, skin reactions (necrosis), or death were recorded on a scale of 4+ to 0.

Pertussis vaccine potency assay. Potency assays were carried out as outlined in Title 21, Code of Federal Regulations, Section 620.4 (1978), using female N:NIH (SW) mice. To compare the relative immunogenicities of vaccines prepared from fresh isolates and derivative strains, a single dose of each vaccine was administered which theoretically should have protected 95% of the animals. An effective dose$_{95}$ (ED$_{95}$) for the U.S. Standard Pertussis Vaccine, Lot No. 7, was estimated to be 0.0125 ml/mouse. This dose of Standard Vaccine was ad-

ministered by diluting the reconstituted vaccine 1:4 and injecting groups of mice intraperitoneally with a volume of 0.5 ml. The vaccine suspension thus diluted read 120 KU (540 nm).

To give an equivalent dose of the test vaccines (based on turbidity), each vaccine was initially adjusted to 170 KU (540 nm). This Klett reading corresponded to one-half the turbidity reading of the reconstituted U.S. Standard Vaccine (330 KU). The test vaccines were then diluted 1:2, and groups of mice were injected intraperitoneally with volumes of 0.5 ml. The test vaccines thus diluted read 70–75 KU (540 nm).

Histamine sensitizing factor (HSF). HSF was assayed by injecting 0.2 ml of each test vaccine intravenously into female N:NIH (SW) mice weighing 20–25 g. Groups of mice were challenged intraperitoneally 3–5 days later with 1 mg of histamine base (histamine diphosphate, Sigma). The numbers of deaths were scored 1 hour and 24 hours after challenge.

Lymphocytosis promoting factor (LPF). Female N:NIH (SW) mice (20–25 g in weight) were injected intravenously with 0.2 ml of the test vaccines. Three to 5 days after injection, blood samples were obtained from the tail vein and white blood counts (wbc/mm^3) were determined using a hemocytometer.

Antibiotic susceptibility testing. Antibiotics were obtained from Sigma Chemical Co, St. Louis, Missouri. Stock solutions of each antibiotic were prepared at a concentration of 2,000 μg [or International Units (IU)] per ml and were stored at −70° C. An exception was ampicillin, which was prepared fresh as needed. Stock solutions of penicillin, methicillin, tetracycline, and streptomycin were dissolved in water, erythromycin and chloramphenicol were dissolved in 95% ethanol; and ampicillin was dissolved in SS liquid medium. Twofold dilutions of each antibiotic were prepared in SS liquid medium, and 1 ml of each dilution was added to 8.5 ml of SS liquid medium, contained in 20 × 175 mm tubes. Each tube was inoculated with 0.5 ml of a 24-hour culture adjusted to 150–175 KU (540 nm). Control tubes containing 9.5 ml of SS liquid medium without antibiotics were also inoculated. Tubes were incubated for 24 hours at 35° C in a rotary shaker (180 rev/min). After 24 hours, each tube was visually inspected for growth, and Klett readings were taken from tubes showing little or no growth. The minimal inhibitory concentration (MIC) for each antibiotic was determined as the lowest concentration of antibiotic resulting in complete inhibition of growth.

After the Klett readings were completed, three to five 0.01 ml replicate samples were subcultured onto BG plates without peptone from tubes with no apparent turbidity. Plates were incubated for 5 days at 35° C and colony counts were performed. The minimal bactericidal concentration (MBC) for each strain was defined as the lowest concentration of antibiotic that resulted in no growth.

Fatty acid sensitivity testing. Fatty acids, obtained from Sigma Chemical Co. were the highest grade available. All fatty acids were in the free acid form, except formic, acetic, and propionic, which were purchased as the sodium salts. Short-chain saturated fatty acids (C_1 to C_5) were dissolved in S/S medium and sterilized by filtration. All other fatty acids were dissolved in 95% ethanol without filter sterilization.

A 5% (v/v) inoculum from 24-hour liquid cultures was used to inoculate flasks containing 50 ml SS medium and various concentrations of fatty acids. Control flasks containing corresponding amounts of ethanol were inoculated for each concentration of fatty acid tested. The concentration of ethanol used did not significantly inhibit the growth in the control flasks. Turbidity was measured for 24 hours using the Klett-Summerson colorimeter (540 nm). Inhibition or stimulation of growth was expressed as the percent of control turbidity measured at 24 hours.

RESULTS

Method for Obtaining Derivative Strains of *B. pertussis*

Four observations were made during the serial passage of fresh isolates to obtain derivatives of *B. pertussis* that would grow on TSA. First, with the use of heavy inocula, each fresh isolate was easily subcultured from BG medium containing 25% blood onto TSA containing 5% and 2% blood. Second, several additional passages on TSA + 2% blood were required before any strain would grow on TSA + 1% blood. Third, consistent growth on TSA without blood was very difficult to obtain. (With each strain, growth on TSA could be initiated only by heavily inoculating small sectors of the TSA plates. After growth on sectors had been established, cells from sectors could be used to inoculate entire plates of TSA. In every case, several additional passages on TSA were necessary before growth was stabilized.) Fourth, once a strain grew

stably on TSA, it was easily maintained during subsequent passages. Such strains typically showed heavy growth 48–72 hours after being transferred to fresh TSA plates.

Morphological and Cultural Properties of Fresh Isolates and Derivative Strains of *B. pertussis*

No differences in cellular morphology could be detected between fresh isolates and derivative strains stained by Gram's method. Typical, small, Gram-negative coccobacilli were seen with each strain. Individual cells appeared slightly larger when grown in SS medium. A larger cell size in liquid medium was not unexpected, since this phenomenon has been reported by other workers (10). Electron microscopy using negative stains revealed no significant differences between fresh isolates and derivative strains.

Although a detailed analysis was not undertaken, the colonial morphology of fresh isolates and derivative strains appeared to be identical. When both strains were grown on BG medium, they formed smooth colonies of heterogeneous size. Heterogeneity in colony size has been described by Cameron as typical for *B. pertussis* (11). Colonies of derivative strains grown on TSA did not have a "rough" colonial appearance.

A difference between fresh isolates and derivative strains was observed when suspensions were made from each strain. Fresh isolates were easily scraped from BG plates and suspended in saline or SS medium. Derivative strains grown on TSA were much more difficult to pick up on a loop, and usually formed clumps when suspended in saline or SS medium. These clumps were broken up only by vigorous vortexing of the suspensions.

Serological Reactions of Fresh Isolates and Derivative Strains

The serological reactions of each fresh isolate and its derivative strain grown in SS medium are presented in Table 1. All strains tested had identical titers by fluorescent antibody staining and reacted similarly with factor-specific antisera. Each strain agglutinated with antisera to factors 1 and 3. [Strain UT 25(16) gave a questionable reaction with Factor 1 antiserum. This strain, when further tested by Dr. Stainer, also failed to agglutinate with his phase I antiserum.]

The pattern of agglutination seen with each fresh isolate and its derivative strain was unexpected. Since all six strains had shown similar reactions by FA staining and factor typing, similar results were expected with the tube agglutination tests. Although the titers were not identical, each fresh isolate agglutinated with all three antisera. However, of the derivative strains tested, only strain UT 26(15) still agglutinated with all the antisera, and it gave a reduced titer to each. Derivative strain UT 25(16) failed to agglutinate with two of the antisera, and strain UT 21(32) showed no agglutination with any of the antisera.

FA staining and tube agglutination tests were repeated using both cells grown in SS medium and cells harvested from solid medium (BG for fresh isolates, and TSA for derivative strains). Both growth conditions produced identical results. Factor

Table 1. Serological Reactions of *B. pertussis* Fresh Isolates and Their Derivative Strains

Strain	FA Titer [a]	Agglutination Titer			Factor typing [c]			Agglutination with Phase I Antiserum (Connaught)
		Antiserum			Factors			
		A	B	U.S. Std.	1	2	3	
UT 21(5)	512	8192	2048	2048	+	−	+ +	NT[d]
UT 21(32)	512	NR[b]	NR	NR	+	−	+ +	NT
UT 25(5)	256	1024	2048	2048	±	+	+ +	−
UT 25(16)	256	NR	NR	64	±?	−	+ +	−
UT 26(5)	256	2048	4096	8192	±	+	+ +	−
UT 26(15)	256	1024	256	1024	±	+	+ +	−

[a] FA titer, Fluorescent antibody titer. The FA conjugate used in the titration was prepared from antiserum A, above.

[b] NR, No reaction

[c] Factor typing was performed by Dr. Dennis Stainer, Connaught Laboratories Limited, Ontario, Canada. The subculture numbers of the strains he tested were UT 21(4), UT 21 (24), UT 25(3), UT 25(15), UT 26(3)) UT 26 (11). The agglutination pattern was judged using the scale: + +, fast; +, slow; ±, very slow; and −, no agglutination.

[d] NT, not tested

typing, which was performed using cells harvested from BG plates, has not been repeated.

Biological Activities of *B. pertussis* Fresh Isolates and Their Derivative Strains

The biological activities of each fresh isolate and its derivative strain are presented in Table 2. A killed cell vaccine, prepared from each strain, was tested for relative immunogenicity and for the presence of HSF and LPF. Live cells of each strain were used to test for the presence of DNT.

Vaccines prepared from fresh isolates of *B. pertussis* were protective, sensitized mice to histamine, and caused elevated white counts at the doses given. Each fresh isolate also produced DNT.

Vaccines prepared from the derivative strains showed variation in biological activities. The vaccine prepared from strain UT 21(32) showed a loss of biological activities when compared with the vaccine prepared from its parent strain, UT 21(5). The vaccine from UT 21(32) afforded little protection to mice at the dosage given, failed to sensitize mice to histamine, and failed to produce an elevated white count. DNT was not detected when live cells of strain UT 21(32) were injected into infant mice.

The vaccine prepared from strain UT 25(15) seemed to show an intermediate loss of biological activities when compared with the vaccine from UT 25(5). Protective activity appeared to be reduced, and HSF and LPF activities were decreased.

DNT was not detected when live cells were injected into infant mice.

The vaccine prepared from derivative strain UT 26(15) was indistinguishable from the one prepared from its parent strain, UT 26(5). Both vaccines afforded the same protection for mice and showed equivalent levels of HSF and LPF. DNT was detected in both strains.

Additional Characterization of *B. pertussis* Strains UT 21(5) and UT 21(32)

Since strains UT 21(5) and UT 21(32) differed so much from each other in serological and biological tests, these two strains were chosen for additional study. Both strains were tested for susceptibility to antibiotics and for sensitivity to saturated and unsaturated fatty acids.

The antibiotic susceptibility pattern of each strain is shown in Table 3. The two strains differed only in their susceptibility to penicillin and erythromycin. Strain UT 21(32) showed increased resistance to both antibiotics when compared with its parental strain, UT 21(5).

These strains were also tested with saturated and unsaturated fatty acids, in concentrations which have previously been shown to inhibit the growth of *B. pertussis* strain 114. (Field, L. H.; Parker, C. D. Effects of fatty acids on the growth of *B. pertussis* in defined medium. Manuscript in preparation.) The effect of saturated fatty acids on the growth of strains UT 21(5) and UT 21(32) is presented in Table 4. No significant differences between the two strains were observed with saturated fatty acids containing fewer than 10 carbons. However, derivative strain UT 21(32) was much more resistant to inhibition by fatty acids containing 10–16 carbons

Table 2. Biological Activities of *B. pertussis* Fresh Isolates and Their Derivative Strains

Strain	Immunogenicity (S/T) [a]	HSF [b] (D/T)	LPF [c]	DNT [d]
UT 21(5)	17/18	9/18	2.3	+
(32)	3/17	0/18	1.2	−
UT 25(5)	13/15	16/18	3.5	+
(16)	11/16	8/16	2.2	−
UT 26(5)	13/16	13/17	3.4	+
(15)	13/15	12/17	3.1	+
U.S. Standard Pertussis vaccine	16/17	NT	NT	NT

[a] S/T, Survivors/total number of mice. A single dose of each vaccine was administered which theoretically should have protected 95% of the animals.
[b] HSF, Histamine sensitizing factor. Results are expressed as deaths/total number of mice challenged.
[c] LPF, Lymphocytosis promoting factor. Results are expressed as the ratio of test groups/saline injected controls.
[d] DNT, Dermonecrotic toxin

Table 3. Antibiotic Susceptibilty of *B. pertussis* Strains UT 21(5) and UT 21(32)

Antibiotic	UT 21(5) MIC[a] (MBC)[b]	UT 21(32) MIC (MBC)
Penicillin	4 (>128)	>128 (>128)
Ampicillin	32 (128)	16 (128)
Methicillin	>128 (>128)	>128 (>128)
Erythromycin	0.12 (2)	2–4 (>8)
Tetracycline	4 (64–128)	4 (>128)
Streptomycin	16 (64)	16 (32)
Chloramphenicol	2 (64)	2 (128)

[a] MIC, Minimal inhibitory concentration (μg/ml; IU/ml for penicillin only)
[b] MBC, Minimal bactericidal concentration (μg/ml; IU/ml for penicillin only)
Values represent the mean of duplicate or triplicate experiments.

Table 4. The Effect of Saturated Fatty Acids on the Growth of B. pertussis Strains UT 21(5) and UT 21(32)

Fatty Acid	Chain Length (carbons)	Conc. a (mM)	% of Control Turbidity UT 21(5)	UT 21(32)
Acetic	2	250	37	43
Proprionic	3	50	31	23
Caprylic	8	7.5	6	4
Nonanoic	9	0.5	25	16
Capric	10	0.25	20	55
Undecanoic	11	0.05	42	97
Lauric	12	0.05	9	86
Tridecanoic	13	0.05	5	88
Myristic	14	0.005	63	94
Pentadecanoic	15	0.005	29	100
Palmitic	16	0.005	13	91

Values represent the mean of duplicate or triplicate experiments.

a Concentrations were chosen which had previously been shown to inhibit the growth of B. pertussis strain 114.

than was strain UT 21(5). The same pattern of increased resistance was evident with the unsaturated fatty acids tested (Table 5). In general, UT 21(32) was no longer inhibited by the unsaturated fatty acids tested, and in fact growth was markedly stimulated by some. No differences were observed between the two strains with palmitoleic acid, however. Arachidonic acid, which was stimulatory to strain UT 21(5), was 2.5 times more stimulatory to strain UT 21(32).

DISCUSSION

Two types of variation have been reported for B. pertussis. The first, generally designated as phase variation, was reported by Leslie and Gardner (1), and later studied by Standfast (2). This type of variation is exhibited by cultures of B. pertussis repeatedly subcultured in vitro and is generally believed to result in irreversible phenotypic changes. The second type of reported variation, modulation,

is induced when cultures of B. pertussis are grown on medium in which NaCl is replaced by $MgSO_4$ or other salts (12,13). Modulation appears to be freely reversible.

We wanted to investigate the variations that cultures of B. pertussis undergo when repeatedly passaged in vitro. So we could draw meaningful conclusions from our data, we took certain precautions. Only fresh clinical isolates of B. pertussis were used as starting strains for serial passages. Careful records of each subculture were maintained. When derivatives were obtained, their properties were compared with those of the parent strain, in the same experiment.

We set out to answer three questions: Is it possible to consistently isolate derivative strains of B. pertussis from fresh clinical isolates that will grow on ordinary nutrient medium containing no blood? If such strains are isolated, how do they differ from intermediate and parental strains? Are such derivative strains the result of mutation, modulation, or both processes? Our results only begin to answer these questions.

We have demonstrated that it is possible to isolate derivatives of B. pertussis that will grow on ordinary nutrient medium (TSA). To date, we have passaged a total of five fresh isolates using this technique and have successfully obtained intermediate and derivative strains from each one. All five strains have shown similar adaptation patterns, although the number of transfers necessary to initiate growth in TSA without blood has varied. In our experience, obtaining growth on TSA with 5% blood or TSA with 2% blood is relatively easy after a few subcultures. This result is in contrast to that reported by Standfast (2). Obtaining growth of the same strains on TSA with 1% blood is more difficult. We have found that obtaining growth on TSA without blood is very difficult and can only be accomplished

Table 5. The Effect of Unsaturated Fatty Acids on the Growth of B. pertussis Strains UT 21(5) and UT 21(32)

Fatty Acid	Chain Length (carbons)	Conc.a (mM)	% of Control Turbidity UT 21(5)	UT 21(32)
Palmitoleic	16:1 (cis-9)	0.0025	40	28
Petroselinic	18:1 (cis-6)	0.0025	22	135
Oleic	18:1 (cis-9)	0.0025	10	157
Elaidic	18:1 (trans-9)	0.0025	11	139
Linoleic	18:2 (cis-9, cis-12)	0.1	18	60
Linolenic	18:3 (cis-9, cis-12, cis-15)	0.1	22	109
Arachidonic	20:4 (5,8,11,14)	0.1	139	251

Values represent the mean of duplicate or triplicate experiments.
a Concentrations were chosen which had previously been shown to inhibit the growth of B. pertussis strain 114.

by heavily inoculating sectors of plates. Once growth on TSA is established, however, it is easily maintained.

The results presented here represent a partial characterization of three fresh isolates and a derivative from each strain that will grow on TSA. Although more complete studies must be done, several differences between the derivatives and their parent strains are evident. First, growth on TSA does not indicate that a pertussis strain has lost all toxic or immunogenic substances. Of the three derivatives we tested, only one showed a uniform loss of protective activity, and it had significantly lower levels of HSF, LPF, and DNT. Whether derivative strains will continue to lose or gain properties with additional transfers on TSA remains to be seen. Preliminary experiments with strain UT 25 have revealed that biological activities that were decreased at passage 16 were demonstrated again at passage 43. Certainly, we must conclude from these results that growth on ordinary nutrient medium is not a sufficient criterion for classifying a strain of *B. pertussis* as degraded or phase IV.

Secondly, serological heterogeneity was evident among the derivative strains we tested. Two of the derivative strains showed significant decreases in agglutination titers. However, all three still reacted to the same titer as their parental strains by fluorescent antibody staining. These results suggest that the fluorescent antibody reaction and the agglutination reactions may be detecting different surface antigens. Whether agglutinating antibody made against whole cells measures the same antigenic determinants as does factor-specific antibody cannot be determined from our results. We must conclude, however, that agglutination reactions cannot be used as the sole criterion for the designation of a *B. pertussis* strain as degraded or phase IV.

Third, when one of the three derivative strains was compared with its parental strain for sensitivity to several antibiotics and fatty acids, significant differences were observed. The derivative strain showed increased resistance to penicillin and erythromycin and was no longer inhibited by saturated fatty acids >10 carbons in length or by most of the unsaturated fatty acids tested. Fresh isolates of *B. pertussis* are inhibited by fatty acids and other substances present in isolation media. Blood, albumin, starch, or charcoal is required to neutralize the effects of these inhibitors. We believe derivative strains can successfully grow on TSA without blood because they have become resistant to the inhibitory

effects of fatty acids and other substances. Perhaps after more strains have been examined, sensitivity to fatty acids may prove to be a useful criterion for describing degraded or phase IV strains of *B. pertussis*.

Many questions are not answered by this study, including whether the differences we have observed are due to mutation, to modulation, or both processes. Certainly, the pattern of loss we have observed in these strains, especially decreased sensitivity to fatty acids, tends to support genetic change, as postulated by Parker (3). Whether biological, serological, and growth properties will change after the strains are passaged on BG or passaged in animals remains to be seen.

Since reference strains do not exist, it is difficult to classify strains of *B. pertussis* as being fully degraded or phase IV. Currently, there is no agreement on which criteria should be used to describe such strains. Derivative strain UT 21(32), which has lost a variety of serological and biological properties, may meet the criteria for a fully degraded *B. pertussis* strain.

These studies have yielded results that may be of value in understanding the biology of *B. pertussis*. Although variation among derivative strains is not understood, we are now in a position to test several hypotheses.

Table 6. Biological Activities of Different Pertussis Vaccines Administered on the Basis of Their Protein Content

Preparation Tested	HSD_{50} [a] (μg)	LPF Activity [b] (μg)	DNT Activity [c]	Pyrogenicity [d] (ng)
Whole cells	23.5	100	0.25	150
Crude ribosomes	53	137.6	0.25	15
Washed ribosomes	250	169.2	2.50	1.5
High salt wash	35	127.1	2.50	150

[a] HSD_{50} = mean histamine sensitizing dose
[b] LPF = lymphocytosis promoting factor; μg required for a twofold increase in wbc/mm³
[c] DNT = dermonecrotic toxin; μg required for a 2+ necrotic response
[d] Nanograms required to raise the temperature of 3 rabbits an average of 0.5° C/animal

ACKNOWLEDGMENTS

REFERENCES

1. Leslie, P. H.; Gardner, A. D. The phases of *Haemophilus pertussis*. J. Hyg. (Camb.) 31:423–434; 1931.
2. Standfast, A. F. B. The phase I of *Haemophilus pertussis*. J. Gen. Microbiol. 5:531–545; 1951.
3. Parker, C. Role of the genetics and physiology of *Bordetella pertussis* in the study of host-parasite relationships in pertussis. Adv. Appl. Microbiol. 20:27-42; 1976.
4. Field, L. H.; Parker, C. D. Pertussis outbreak in Austin and Travis County, Texas, 1975. J. Clin. Microbiol. 6:154–160; 1977.
5. Stainer, D. W.; Scholte, M. J. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. J. Gen. Microbiol. 3:211–220; 1971.
6. Eldering, G.; Eveland, W. C.; Kendrick, P. L. Fluorescent antibody staining and agglutination reactions in *Bordetella pertussis* cultures. J. Bacteriol. 83:745–789; 1962.
7. Hebert, G. A.; Pittman, B.; McKinney, R. M.; Cherry, W. B. The preparation and physiochemical characterization of fluorescent antibody reagents. Atlanta, Georgia: Center for Disease Control; 1972.
8. Pittman, B. *Bordetella*. Lennette, E. H.; Spaulding, E. H.; Truant, J. P., eds. Manual of clinical microbiology, 2nd edition. Washington, D. C.: American Society for Microbiology; 1974: 308–315.
9. Manclark, C. R.; Urban, M. A.; Summers, E. P.; Vickers, J. H. Assay of pertussis vaccine toxicity by a rat-paw-oedema method. J. Med. Microbiol. 10:115–120; 1977.
10. Munoz, J. J.; Bergman, R. K. *Bordetella pertussis:* immunological and other biological activities. New York: Marcel Dekker, Inc.; 1977.
11. Cameron, J. Problems associated with the control testing of pertussis vaccine. Adv. Appl. Microbiol. 20:57–80; 1976.
12 Lacy, B. W. Antigenic modulation of *Bordetella pertussis*. J. Hyg. (Camb.) 58:57–93; 1960.
13. Parton, R.; Wardlaw, A. C. Cell envelope proteins of *Bordetella pertussis*. J. Med. Microbiol. 8:47–57; 1975.

Physical, Chemical, and Serological Properties of the Glutamine Synthetase of *Bordetella pertussis*

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ABSTRACT

Glutamine synthetase (GS), an important enzyme in microbial metabolism, has been demonstrated in *Bordetella pertussis* and *Bordetella parapertussis* in ultrasonic disintegrates and preparations of protectosomes. Polyacrylamide gel electrophoresis of fractions from ultrasonic disintegrates showed 10–12 protein components. Polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate (SDS) with the GS first treated with dithiothreitol and iodoacetic acid showed three protein bands in the GS fraction.

Comparison of GS SDS gel patterns with reference proteins showed molecular weights of 600,000 and 50,000. These values are similar to those obtained for intact *Escherichia coli* GS and its subunits as well as those reported by Sato for *B. pertussis* protective antigen.

Glutamine synthetase activity was retained at 4° C for 1 month; at 36° C for 3 days activity was 50%. After incubation for two hours at 70° C and 75° C, remaining activity was 50% and 14%, respectively. Only 7% activity remained after 30 minutes incubation at 80° C.

Immunodiffusion analysis demonstrated a relationship between the GS of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*. In addition, the glutamine synthetase from these species was markedly inhibited by *B. pertussis* hyperimmune globulin (rabbit).

The glutamine synthetase (GS) (EC 6.3.1.2) of *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica* was first isolated in our laboratory from ultrasonic disintegrates. Glutamine synthetase was also prepared from *B. pertussis* protectosomes, which are structures associated with the development of protection against pertussis as measured in the mouse potency test.

During attempts to isolate GS from *B. pertussis* we tried to retain the morphological integrity and immunological activity of the protectosomes, but did not succeed in producing a highly purified enzyme preparation. The GS activity of the isolated preparations was on the order of 702 to 1,780 micrograms of γ-glutamylhydroxamate per mg per hour as measured by the method of Lavintow as described by Woolfolk et al. (1). The molecule of GS of *B. pertussis* was linked with seven to eight adenyl-groups (i.e., it was partly adenylated). All enzymatically active fractions obtained from ultrasonic disintegrates of *B. pertussis* also had mouse protective activity. These fractions were electrophoresed in 4% polyacrylamide gel in Tris-glycine buffer, pH 8.1. Twelve protein components were demonstrated

when stained with Amido Schwartz. Gel protein patterns of GS preparations from three *Bordetella* species are presented in Figure 1. They differ in the position and number of minor components. However, two intensely staining bands, a) which migrates
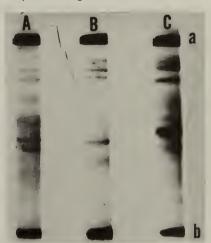

Figure 1. Polyacrylamide gel electrophoresis of GS isolated from *B. parapertussis* (A), *B. pertussis* (B), and *B. bronchiseptica* (C). Gels contained 100–200 μg protein. Electrophoresis was carried out in 0.025 M Tris-glycine buffer, pH 8.9, at 5 mA per gel (200 V). See text for explanation.

slowly, and b) which migrates rapidly, are present in all preparations. Prior to polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate, the preparations were treated with disulfide bond splitting reagents. About 10 to 20 μg protein was treated for 1 hour at 37° C with a mixture containing 0.1% dithiothreitol, 0.1 M iodoacetic acid, 0.1% sodium dodecyl sulfate in 0.025 M Tris-glycine buffer, pH 8.9. Reference proteins (ferritin, catalase, bovine serum albumin; Servo Fein Biochemica, Heidelberg) were used in all assays. Three pronounced protein bands were found. One showed a relative electrophoretic mobility of 0.159 relative to bromphenol blue. The other two bands had similar electrophoretic mobilities of about 0.970. Molecular weights were calculated to be approximately 600,000 and 50,000, respectively. These results agree with those published by Stadtman and Ginsburg (2) for *Escherichia coli* GS and its subunits. Sato (3) has reported similar molecular weights for *B. pertussis* protective antigen.

The stability of *B. pertussis* GS was determined at temperatures from 4° C to 80° C (see Fig. 2). Enzymatic activity was reduced with increased temperature (1), but GS was found to be relatively resistant to heat. After 3 days at 36° C, 50% activity was maintained. Heating for 2 hours at 70° C and 75° C reduced activity by 50% and 85% respectively. At 80° C the activity decreased by 93% after 30 minutes incubation, and complete inactivation occurred after 60 minutes incubation. Enzymatic activity was retained in full after storage of GS for 1 month at 4° C without any stabilizer.
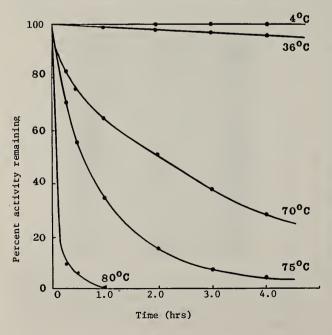
To characterize the serological properties of GS and study the antigenic relationship of *B. pertussis* GS with that of *B. parapertussis* and *B. bronchiseptica*, hyperimmune γ-globulin was prepared from the antisera of rabbits immunized with *B. pertussis* GS. Enzyme was mixed with an equal volume of Freund's Complete Adjuvant (Calbiochem) and injected into a rabbit (one injection in the footpad and three intramuscular injections in the thigh totaling 13.6 mg of protein) at 10-day intervals. The rabbit was bled 10 days after the last injection. γ-Globulin was used for immunodiffusion in 0.8% agarose gel and for inhibition studies of GS activity with enzyme preparations of all three *Bordetella* species. As illustrated in Figure 3A, gamma globulin produced three to four precipitin bands with preparations from the three species of *Bordetella*. Two bands of identity and one band of partial identity were observed. The spurs formed are specific for GS isolated from *B. pertussis*. To identify the band specific for the enzyme, immunodiffusion was performed with both unheated and partially heat-denatured preparations of *B. pertussis* GS. Their residual enzymatic activity was 100%, 50%, and 90% respectively. The results of the reaction (Fig. 3B) showed that the most inactivated preparation (in well 5), containing the smallest number of antigenic determinants, produced the band that crossed the bands made by the unheated (well 4) and partly inactivated (well 6) preparations forming spurs. From these results, we assume that the intense precipitin band is specific for GS. Accordingly, we suggest that the intense precipitin band of identity formed by the GS of all three species *Bordetella* (Fig. 3A) indicates antigenic homology between the

Figure 2. Influence of temperature and time on glutamine synthetase activity.

Figure 3. Immunodiffusion of GS of the three *Bordetella* species. The central wells contained 240 μg of anti *B. pertussis* γ-globulin. Different GS preparations (40 μg of protein) are present: 3A intact from *B. bronchiseptica* (1), *B. pertussis* (2), *B. parapertussis* (3); 3B from *B. pertussis*—unheated (4), heated for 10 minutes at 80°C (5) and 10 minutes at 70° C (6).

GS of *B. pertussis, B. parapertussis,* and *B. bronchiseptica.*

The relationship between the GS of three *Bordetella* species was also demonstrated by neutralization of enzyme activity by a modification of the method described by Tronick, et al. (4). Glutamine synthetase from each species of *Bordetella* (170 to 200 μg protein in 0.1 ml) was mixed with an equal volume containing various amounts of anti-GS γ-globulin (1,200 μg to 0.94 μg of globulin in 0.01 M Tris-hydrochloride buffer, pH 7, containing 0.01 M magnesium chloride). The residual GS activity was measured in each sample after incubation for 1 hour at 36° C and overnight at 4° C. As a control, normal rabbit γ-globulin was used in similar experiments, and showed no inhibition. Six hundred μg of anti GS γ-globulin inactivated the GS activity of *B. pertussis, B. parapertussis,* and *B. bronchiseptica* by 88.1, 78.2, and 77.0%, respectively. These results are consistent with the antigenic homology of GS from other Gram negative bacteria (4) and also indicate that the protective function of protectosomes is not related to GS, since the GS from *B. parapertussis* and *B. bronchiseptica* do not induce immunity to intracerebral challenge with *B. pertussis* in the mouse potency test.

REFERENCES

1. Woolfolk, C. A.; Shapiro, B. M.; Stadtman, E. R. Regulation of glutamine synthetase. I. Purification and properties of glutamine synthetase from *Escherichia coli.* Arch. Biochem. Biophys. 116:177–192; 1966.
2. Stadtman, E. R.; Ginsburg, A. The glutamine synthetase of *Escherichia coli:* structure and control. Boyer, P., ed. The Enzymes. New York: Academic Press, Inc.; 1975:737–773.
3. Sato, Y. Isolation and some properties of the protective antigen from *Bordetella pertussis.* Symposium on pertussis vaccine, Symposia Series in Immunobiological Standardization, 13:214–220; 1970.
4. Tronick, S. B.; Ciardi, J. E.; Stadtman, E. R. Comparative biochemical and immunological studies of bacterial glutamine synthetase. J. Bacteriol. 115:858–868; 1973.

DISCUSSION OF PART 2

DR. EZZELL: I would like to ask Dr. Hewlett and Dr. Wardlaw, who have run the adenylate cyclase studies, whether there is activity of this enzyme at 24° C.

DR. HEWLETT: We have done temperature studies of the enzymes both with and without activator. There is measurable enzyme activity, although it is reduced at 24° C.

DR. EZZELL: What percentage activity do you see at 24° C?

DR. HEWLETT: About 50%.

DR. EZZELL: Is it possible that we may be thinking of this organism incorrectly? It has been assumed that phase I cells are the natural, wild-type strain in nature. Dr. Wardlaw has shown that the pertussis grown at low temperatures resembles a phase IV strain in many respects. Is it possible that the organism in nature is actually a C-mode cell since conditions (temperature, nutrient) may not be optimal? Could it be that when these organisms are then introduced into the warm respiratory tract of a susceptible host they are induced by increased cyclic AMP activity to a virulent form?

DR. PARKER: Would anyone care to comment on Dr. Ezzell's very interesting suggestion?

DR. LASALLE: Where would these saprophytic or avirulent forms be found in nature?

DR. EZZELL: I don't know; but it may be that some animal, possibly avian, host carries the C-mode organisms. Under certain circumstances, one can envision the introduction of these organisms into a susceptible human host, resulting in disease. One hesitates to draw the obvious analogy to the current theories of transmission of Legionnaires' Disease.

DR. LASALLE: Do we know of any instance where any *Bordetella* species have been found in soil or water?

DR. EZZELL: No.

DR. J. B. ROBBINS: Has anyone compared the different phases of pertussis for the presence or absence of the fimbriae mentioned by Dr. Sato? Are avirulent nonimmunogenic strains fimbriae positive or negative?

DR. PARKER: Some of our derivative strains have lost hemagglutinating activity. Perhaps Dr. Sato would care to comment. I don't have his Tohama phase III strain. Dr. Sato, is your Tohama phase III strain avirulent, and does it have the hemagglutinin?

DR. SATO: It is avirulent and it lacks hemagglutinin and fimbriae.

DR. BERRY: Dr. Hewlett, do you know whether the activation of the adenylate cyclase in *B. pertussis* resembles that for *Vibrio cholerae* and for *Escherichia coli*? Does it require NAD and ATP? Is there less enzyme in phase IV (or C-mode) organisms and is it more difficult to activate?

DR. HEWLETT: In answer to the first question, we have looked at the possibility that the activator might be a protease and have found no evidence for this in several studies using low levels of trypsin and chymotrypsin.

We have looked very specifically at the effect of cholera toxin on the *Bordetella pertussis* enzyme in the presence of NAD and the various other factors that are required for mammalian cyclase, and there was no influence on the enzyme activity.

I have not looked at C mode cells myself. In the phase IV strains that we have looked at, we are unable to quantitate enzyme protein. Thus the low activity observed may reflect either low levels or inactive enzyme.

DR. DOBROGOSZ: Dr. Parker and Dr. Hewlett, this question concerns strains UT–21, 25, and 26. These are fresh isolates. Do they grow on synthetic media?

DR. PARKER: They grow in our laboratory, but Dr. Hewlett has had difficulty.

DR. DOBROGOSZ: Well, it is appropriate that I ask you both the question: What media are you using?

DR. PARKER: We are using the modified Stainer-Scholte medium (Stainer, D. and Scholte, M. J. Gen. Microbiol., 63: 211, 1971) which has the Tris reduced to one-quarter. We also use acid-cleaned glassware.

DR. DOBROGOSZ: And they do grow?

DR. PARKER: Yes. We have made clinical isolations directly on Stainer-Scholte medium (solidified with purified agarose). *Bordetella pertussis* from the nasopharynx grows on Stainer-Scholte medium if you use agarose rather than agar.

DR. DOBROGOSZ: Dr. Hewlett, the very simple technique that we use is to put bovine serum albumin (BSA) in the medium. We have no trouble with initiation of growth from single cells on agar surfaces, as long as we incorporate the BSA.

DR. HEWLETT: Yes, I think that the problem was alluded to by Dr. Parker. We may have problems with our glassware. We have not tried the BSA as you've indicated, but I suspect that that would help.

DR. DOBROGOSZ: Does anyone have trouble growing these fresh isolates if BSA is in the medium? Is this a "universal protector" for freshly isolated *Bordetella* strains, or it is not?

DR. PARKER: I'm not sure. We find inhibition of fresh isolates if we use either a commercial BG base or if we add peptone to the BG. For our antibiotic and fatty acid studies, we have been unwilling to put albumin in the medium because we can't quantitate the binding of the inhibitor to the albumin.

I agree with you that for routine use, the addition of albumin, charcoal, starch, Dowex, or red blood cells is useful, presumably to bind some of the inhibitors.

DR. EZZELL: When phase I strains have been stored in a medium at 4°C for several weeks, they are indeed stimulated by BSA and gelatin.

DR. COWELL: Dr. Ezzell, you said you added exogenous cyclic AMP to phase IV cells with no effect. Did you try adding it to C mode grown cells?

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DR. EZZELL: Yes. We do not see reversion to a phase I type. Magnesium may interfere with the uptake of cyclic AMP, but even with levels of up to 10 millimolar we have not seen any effect.

DR. ASKELÖF: Dr. Hewlett, is it possible that this activator of yours is an inactivator binding protein and that you lose your enzymatic activity during purification? Have you tried any other things like charcoal, ion exchange resins, or BSA to see if they could raise the activity?

DR. HEWLETT: I hesitate to list all the various protein compounds that we have gone through to look for activator activity! We have examined a number of mammalian tissues, including liver, muscle, tracheal mucosa, and erythrocytes. All these had activator activity and we became suspicious that there might be some nonspecific protein protective effect. We looked at a number of compounds such as protease-inhibitors, albumin, ovomucoid, and alpha-casein. These had a small, but in no way comparable, stimulatory effect on the whole cell cyclase activity.

DR. ASKELÖF: But still you cannot exclude an inhibitory binding protein and an inactivation of your enzymatic activity during purification.

DR. HEWLETT: We considered the possibility of an inhibitor, or that the activator was inactivating an inhibitor. However, the adenylate cyclase activity of the whole cell is 2.1 nanomoles of cyclic AMP/min/mg of protein. When activator is added, this activity goes up 300-fold. When the cyclase is purified, there is a tenfold increase in the specific activity, together with a loss of responsiveness to activator. If there were an inhibitor present in the whole cell, one would expect that as inhibitor is lost during purification the adenylate cyclase activity would approach a value of 600—the maximum activity achieved when whole cell associated enzyme is activated.

DR. CHANDLER: Dr. Wardlaw, what concentrations of nicotinic acid will give you your effects? Second, have you tried nicotinamide and NAD to see if they will also cause the loss of the protein bands and the loss of the biologic activities? What sort of information do you have on the possible mechanism of the nicotinic acid effects?

DR. WARDLAW: The level of nicotinic acid we used is 500 micrograms per ml as compared with one to five micrograms per ml in the normal X-mode medium. We have therefore used 100 to 500 times the normal level. One of the remarkable things is that a high level of nicotinamide does not produce modulation, and we have no explanation for this.

DR. PEETZ: I have some data for Dr. Ezzell. We screen a lot of dogs for *Bordetella bronchiseptica,* and we receive many animals which have full-blown infection with *B. bronchiseptica* but which exhibit no overt disease.

Similarly, we have virulent porcine strains which are totally avirulent in the dog. We have observed a strain virulent in dogs and cats which produced a severe respiratory infection in the spouse of one of our laboratory workers although she had been previously immunized with DTP.

DR. PARKER: As I understand it then, you're saying there is host specificity with *Bordetella bronchiseptica?*

DR. PEETZ: Precisely.

DR. CAMERON: I should like to put a question to Dr. Ezzell. I believe in one of your early slides you suggested that the ability to induce hemolysis would be a definitive character of phase I strains. Was that correct?

DR. EZZELL: Of the phase I strains that we have seen, that is one of the characteristics that we use with cultures on BG. I have heard reports of degraded strains also being hemolytic.

DR. CAMERON: It has been my experience with a considerable number of clinical isolate plates that pertussis colonies can be hemolytic or nonhemolytic. In some cases a mixture of both is seen on the isolation plate.

Another point I should like to make in relation to Dr. Dobrogosz's question is that erythrocytes are the essential component of blood in assisting the growth of pertussis. You can dispense with the serum and plasma.

I have found it advisable, when trying to build the definitive picture as to whether or not a strain is hemolytic, simply to take the erythrocytes (equine, ovine, bovine, caprine, lapine), wash them well, and incorporate them in the medium. I do not recall anyone's suggesting that blood itself is inhibitory, but blood itself can be inhibitory in some circumstances. This is evident when growth of cultures is delayed with a different lot of blood.

DR. SCHUH: We have developed a solid medium that does not contain blood. The composition of this medium is given below: Sulfuric acid hydrolyzed casein (10 g); $NaCl_2$ (7.3 g); KH_2PO_4 (0.5 g); $MgCl_2 \cdot 6H_2O$ (0.04 g); $CaCl_2$ (0.1 g); $FeSO_4 \cdot 7H_2O$ (0.01 g); glutathione (0.01 g); nicotinamide (0.02 g); starch (1.25 g); Dowex (1.0 g); distilled water to a liter; pH 7.1 to 7.2.

The addition of yeast dialysate (5% v/v) and catalase (0.3 ml/liter medium, i.e. approx. 75,000 units) provided a medium comparable in sensitivity to Bordet-Gengou which, even after 70 years in existence, remains a masterpiece of the French kitchen.

Our medium has the advantage of being low in antigenic protein, inexpensive and easily reproducible.

We have also analyzed the activity of acetate and lactate on growth using this medium. In our hands, acetate has been inactive or inhibitory. Lactate was marginally stimulatory at low concentrations (optimum approx. 0.08%).

DR. STAINER: In the early developmental work on the synthetic medium, I was interested in the effects of varying the medium composition on the biological properties of *B. pertussis.* In the accompanying figures the notations G and P refer to the basal levels as previously published (Stainer, D. and Scholte, M., J. Gen. Microbiol. 63:211; 1971). Thus 12G and 12P indicate, respectively, 12 times the basal levels of glutamate and proline. Three parameters were examined, namely mouse-protective antigen (MPA), histamine-sensitizing factor (HSF) and heat labile toxin (HLT). Figure 1 shows the effect of increased glutamate levels on MPA and HSF levels and it is clear that significant elevations were achieved. Similarly in Figure 2 the effects of increased proline levels were noted and here the effects were even more
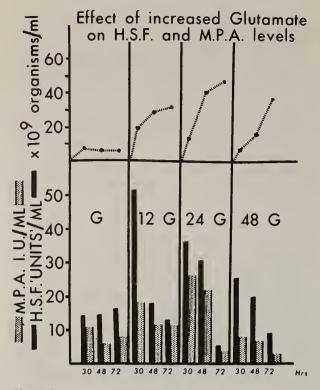
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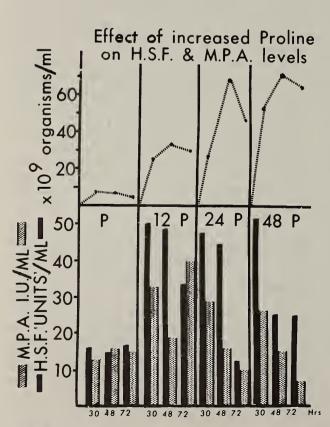
Figure 2

dramatic. Figure 3 illustrates the effects of both increased glutamate and proline on HLT levels at different stages of growth. The decrease in the LD_{50} values indicated an increased amount of HLT depending on medium composition.

Thus by a relatively simple omission of an amino acid or by altering the concentration of an amino acid, it is possible to greatly affect some of the biological parameters of *B. pertussis*. In light of the work presented at this session, these old unpublished results can now be fitted into current thinking.

DR. BEMIS: Dr. Ezzell, you observed a decrease in the outer membrane protein in *B. parapertussis* and *B. bronchiseptica* similar to that observed in *B. pertussis*. Was that correlated to a similar phase difference? If so, how do you define phase variation in the other species?

DR. EZZELL: We have found that rough strains of *B. bronchiseptica* have lost the two proteins which correlate closely to Dr. Wardlaw's proteins, and which are present in smooth strains of *B. bronchiseptica*.

For *B. parapertussis*, we also noted similar proteins in smooth strains, but we did not have any rough strains for comparison. However, we did grow *B. parapertussis* in a high magnesium medium and again we lost the same two proteins.

DR. BEMIS: Have you observed any biological differences in the smooth and rough strains of *B. parapertussis* or *B. bronchiseptica*?

DR. EZZELL: The smooth strains of *B. bronchiseptica* are hemolytic and the rough strains are nonhemolytic on BG.

DR. MUNOZ: I would like to make a short comment directed to Dr. Wardlaw. I think that the extra membrane proteins he finds in the X-mode cells cannot represent all the active proteins, because the molecular weights are too small. I don't know of any work reporting HSF or LPF activity of proteins of 28–30,000 molecular weight.

Perhaps treatment with SDS degrades or separates peptides, which are active only when combined. Dr. Morse has shown this with his LPS, and Dr. Ui has also shown this with his islet-activating protein.

Dr. Ezzell pointed out that if we could isolate these

Figure 3

bands, we could test for their biological activity. I would predict these bands would be completely inactive.

DR. EZZELL: Treatment with SDS and β-mercapto-ethanol will totally dissociate active protein complexes into basic subunits. What we saw on these gels were undoubtedly not the entire proteins.
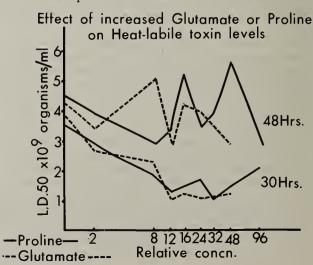
The lowest weight protein which would appear on our gels was about 12,000 molecular weight. Perhaps we should repeat these experiments using conditions which would allow us to observe proteins of a molecular weight below 12,000. This might allow us to detect other phase I unique proteins which when added to the phase I proteins reported thus far would have a molecular weight totaling that of the HSF/LPF proteins.

DR. BLASKETT: I would like to make two short comments and ask Dr. Novotny a question. First, I was pleased to hear that the *Bordetella* species are very much more closely related than we have hitherto believed. I was horrified to read in a diagnostic bacteriology book (I think it was Cowan and Steel), that on nutritional grounds, we had *Bordetella bronchiseptica, Acinetobacter* and *Alkaligenes bronchisepticus*. I felt on antigenic grounds the three *Bordetella* species were related; I am glad to learn that on genetic grounds they are closely related.

Second, Dr. Ezzell raised the question of whether C-mode organisms could become X-mode organisms in vivo. Some years ago my colleague Lawrie Fulton and I did some work which suggested that they do. We made X-mode and C-mode vaccines, and immunized mice. We then challenged them with live X-mode and C-mode organisms. (The C-mode organisms were naturally very much less virulent than the X-mode and the challenge dose was larger.) The interesting thing was that the C-mode vaccines did not seem to protect well against the C-mode challenge, but the X-mode vaccines did. Our interpretation of this was that the few C-mode organisms that survived the host defenses became X-mode organisms. That was why the C-mode vaccine did not protect, but the X-mode vaccine did.

May I now ask Dr. Novotny about unbalanced cultures susceptible to loss of potency when mixed with Alhydrogel? What happens with the unbalanced cultures? Are they stable to Alhydrogel, or do they also lose potency?

Are the balanced cultures stable when stored at elevated temperatures (22° C and 37° C)? What happens to the unbalanced ones when they are similarly stored?

DR. NOVOTNY: Yes, the balanced cultures (those which have full agglutinability and which look smooth in thin section) are not influenced by the presence of Alhydrogel.

The unbalanced cultures are very labile even at 4° C. They lose antigenicity at an unpredictable rate and are unsuitable for vaccine use. The trouble is that very often a producer makes a vaccine and there is some doubt about potency. When this is retested, the reproducibility depends on whether the culture was balanced or unbalanced.

DR. BLASKETT: So, you would say that if the cultures are unbalanced, the resulting vaccines would tend to lose their protective potency more rapidly at elevated temperatures?

DR. NOVOTNY: Not only at elevated temperatures but also at 4° C. Why this is, I don't know.

May I comment on adenylate cyclase? There seems to be confusion. There were tables showing that no adenylate cyclase was detectable. A bacterium which is able to grow in distilled water contaminated with a few amino acids probably always produces adenylate cyclase. The point is that it is regulated by feedback mechanisms. When there is overproduction of an enzyme or a product, it is likely to be due to an abnormal feedback mechanism. Adenylate cyclase overproduction could be an example.

DR. PARKER: Professor Zakharova, not knowing much about nitrogen metabolism in *B. pertussis*, I was interested that you had been studying glutamine synthetase. Do you have comments regarding the levels of this enzyme, its substrates, its products, or the associated enzymes? I am particularly interested in differences between *B. pertussis, B. parapertussis* and *B. bronchiseptica* cells.

DR. ZAKHAROVA: We have not studied the metabolic role of glutamine synthetase. We have only detected it in microbes. The level of GS in protectosomes of *B. parapertussis* was a little higher than in *B. pertussis* phase I.

DR. PEETZ: I have a comment for Dr. Ezzell. We have been studying the genetics of *B. bronchiseptica*. We have noted consistently that phase IV or degraded cells are much more resistant to detergent lysis than are the phase I cells, and especially those detergents that act on the inner membrane. This would indicate a definite structural difference between the two.

DR. CSIZER: I have a question for Dr. Novotny. You reported the instability of some of your vaccines when Alhydrogel was added. Was it a real loss of potency due to the effect of Alhydrogel? If these so-called unbalanced vaccines have higher toxicity, the toxicity-promoting activity of Alhydrogel could suppress the actual immunogenicity of these vaccines in mice.

DR. NOVOTNY: We do not know why, for example, the addition of Alhydrogel can cause a drop in protective units from 20 to 1 per ml. It occurs in vaccines prepared from cells grown in defined and complex media.

DR. STAINER: Have you tried other aluminum compounds?

DR. NOVOTNY: The drop is less pronounced with aluminum phosphate, but with Alhydrogel it is often so critical that it makes the suspension completely useless.

DR. SCHUCHARDT: I would like to comment on what Dr. Novotny has said. Several years ago we saw the same thing. We started out with a bulk vaccine containing 18 PU/ml. When formulated and retested it gave a value of 2 PU/ml. After storage for a further month it assayed at 6 PU/ml and after 3 months, it assayed at 9 PU/ml. After we had completed 18 tests, it averaged 12. So this appears to be a reversible thing.

We took the same vaccine, adjusted the pH up to 8.5 and assayed it. We brought a portion of it back to pH 5.8, and assayed. The original vaccine gave 3 PU/ml; the vaccine at pH 8.5 gave 18 PU/ml; the vac-

cine in which the pH had been raised and lowered again assayed at 12 PU/ml.

DR. CAMERON: I should also like to confirm what Dr. Novotny has said. When I was working at Wellcome myself, we demonstrated the effect of Alhydrogel in depressing the antigenic response to individual serotypes.

Why are we putting an adjuvant in pertussis vaccine, and what do we expect of this adjuvant? By definition, if an adjuvant enhances the response to an antigen, we know that in the case of diphtheria and tetanus toxoids, the slopes of the dose response curves are altered to such a degree that you cannot compare them in the same assay. Yet we are perfectly happy to continue testing adsorbed pertussis vaccines against a known non-adsorbed reference vaccine. This is just a contradiction in terms if adjuvant is doing anything at all.

DR. CSIZER: The same phenomenon was reported in the case of cholera and adsorbed cholera vaccines. The adsorbed cholera vaccines gave a lower mouse-protective activity than the nonadsorbed ones when tested against the unadsorbed standard vaccine.

DR. STAINER: I would challenge that statement. We found that cholera vaccines in the presence of aluminum phosphate gave elevated protective units in the mouse potency test.

DR. LASALLE: I would like to go back to the epidemiology question and ask if anyone has any evidence whatsoever that the B. bronchiseptica can play a role in the epidemiology of pertussis, either by adaptation, by hybridization, through plasmids, or in any other way?

DR. PARKER: I am not aware of any evidence. If any of the experts here have comments, I would invite you to make them.

DR. NOVOTNY: We have two strains of Bordetella bronchiseptica that were isolated from whooping cough in children. They do not differ significantly, say, from other strains of B. bronchiseptica from pigs or dogs. This indicates that it is possible to have B. bronchiseptica pertussis clinically.

DR. STEWART: I can't comment with any special microbiological knowledge about B. bronchiseptica, but I think, as an epidemiologist, I should say that what we call whooping cough is a syndrome, and that the symptoms of whooping cough that lead to a case being identified as whooping cough and reported as whooping cough can arise from a variety of causes, if by causes we mean the isolation of particular pathogens.

We will be talking about this later, but I think it is important to realize that not only B. bronchiseptica, but a number of other organisms, have been quite firmly associated with the symptoms of what we call whooping cough, and also that this happens not only in children but also in adults.

Now, this is a matter of considerable difficulty, to sort out all those things, and I am afraid that some of the exactness which we have in defining particular protein components of the organism is not obtained in those differentiations of clinical diagnosis. But it does mean that we can answer the question to this extent: that other organisms than Bordetella pertussis are concerned, are associated with a whooping cough-like syndrome, and it may well be that B. bronchiseptica is one such.

DR. EZZELL: As most of you probably know, some batches of fluorescent antibody (FA) to B. pertussis can cross-react with B. bronchiseptica and B. parapertussis. I wonder how many B. bronchiseptica infections have been erroneously designated as pertussis because they fluoresce with the B. pertussis conjugate.

DR. PARKER: I might ask Ms. Field to comment on how to make an FA conjugate if anyone is interested, so that you can avoid the cross-reactivity problem.

MS. FIELD: It is difficult; you must begin with a rabbit that has not been infected with B. bronchiseptica and thus has no pre-existing B. bronchiseptica antibodies before immunizing with B. pertussis.

DR. EZZELL: The problem is that in our hands some batches of the current pertussis FA serum from CDC in Atlanta do cross-react with B. bronchiseptica and B. parapertussis.

DR. PARKER: I think our sera cross-react at only a very low dilution, but we dilute them past this point in order to do fluorescent antibody tests. Nevertheless, there is certainly a need for a standardized FA antibody for pertussis diagnosis.

DR. LASALLE: Perhaps it is possible that isolates of B. bronchiseptica have been mistaken for pertussis because of this cross-reactivity of the fluorescent antibody reagents which have been used in many places without dilutions, and without awareness of this cross-reactivity.

[Dr. Broome has made the following editorial comments regarding the FA reagent from the CDC: 1) the FA reagent is designed for use with fresh clinical isolates of B. pertussis only; 2) it is not clear from Dr. Ezzell's remark whether he diluted his FA antiserum and titered it against B. pertussis, B. parapertussis, and B. bronchiseptica.]

Part 3. BIOLOGICALLY ACTIVE COMPONENTS OF *Bordetella pertussis*

Chairman: *J. J. Munoz*
Rapporteur: *R. K. Bergman*

Biological Activities of *Bordetella pertussis*

J. J. Munoz and R. K. Bergman

ABSTRACT

Many biologically active substances have been isolated from *B. pertussis,* Among these are the heat-labile dermonecrotic toxin, an endotoxin, various agglutinogens, hemagglutinin, pertussigen and many lesser known antigenic substances. Dermonecrotic toxin, endotoxin, and pertussigen are the most active biologically. A great number of changes occur in experimental animals treated with pertussis vaccines or with extracts from *B.pertussis* cells. Several of these responses are: increased susceptibility to various forms of shock; increased circulating leukocytes and lymphocytes; increased immunoglobulin production, especially of the IgE class; increased susceptibility to autoimmune diseases; increased resistance of mice to experimental infection with *B. pertussis;* and many other responses usually associated with adjuvant action and with enhanced susceptibility to shock.

The acute and highly lethal effects of *B. pertussis* are most likely due to the dermonecrotic toxin and the endotoxin. Their modes of action, however, are largely unknown. Most of the other effects apparently are due to the action of pertussigen: its adjuvant activity and/or its blockade of some functions of epinephrine. It is difficult to interpret how pertussigen induces leukocytosis and lymphocytosis by either of these proposed mechanisms. It may be that changes in lymphocyte surface membranes result in redistribution of these cells, as has been postulated by Morse, although permeability of capillary beds may also be important. The ability of pertussigen to enhance development of experimental allergic encephalomyelitis and increase resistance to intracerebral infection with *B. pertussis* may be due to an increase in the response to the respective antigen and an interference with the function of epinephrine that controls permeability of capillaries of the central nervous system. The possible role of other *B. pertussis* cell antigens in immunity to experimental infection and immunization of man against pertussis is discussed.

Bordetella pertussis produces various biologically active substances, of which dermonecrotic toxin, agglutinogens, endotoxin, hemagglutinin, and pertussigen are the most important. Whole *B. pertussis* cells given to animals induce many effects that can be grouped into three fundamental actions: increased susceptibility to various forms of shock; increased immunological response to antigens given with the cells; and toxic actions. Many physiological changes produced by the cells, such as leukocytosis and lymphocytosis, increased production of insulin, proteinemia, and hypoglycemia may be the result of the action of some of the biologically active substances listed. The most obvious toxic actions are due to dermonecrotic toxin and endotoxin, although pertussigen also has toxic properties of its own and accounts for many of the actions of pertussis vaccine.

Some active factors have been purified, but their exact chemical compositions are not well known. The dermonecrotic toxin is a protein. Agglutinogen 1 and pertussigen are probably proteins also. The endotoxin is a lipopolysaccharide.

The exact modes of action of dermonecrotic toxin and endotoxin are unknown. Pertussigen seems to act by blocking some action of epinephrine, but the exact way in which this takes place is not clear.

Many problems remain unsolved. The most important are determination of exact chemical nature of various active substances; determination of exact mode of action of these substances; the role of the various substances in protection against whooping cough; the genetic control of these active substances; and the role of cultural conditions on production of biologically active substances.

The biological activities of *Bordetella pertussis* are well known. Many reviews (1–8) and a monograph (9) have been published giving detailed information on this topic, so we will not describe these activities in detail but will attempt to show that only a few substances isolated from the bacterial cell are responsible for them. We will also discuss the mode of action of these substances.

The biologically active substances that have been associated with *B. pertussis* cells are shown in Table

1. Shortly after discovering *B. pertussis,* Bordet and Gengou (10) demonstrated that this bacterium produced a dermonecrotic toxin that could be extracted from dried cells by grinding with crystals of sodium chloride and then dissolving the material with water to a final salt concentration of 7.5%. Given intraperitoneally, this preparation killed guinea pigs and rabbits, and given subcutaneously, it produced a hemorrhagic edema which became a purple-black necrosis within 24 to 48 hours. These observations were confirmed and expanded by various workers (5, 9). Dermonecrotic toxin was a poor antigen when extracted but became more antigenic after being converted to a toxoid by treatment with formalin (5). The toxin is a protein that can be inactivated in 10 minutes at 56° C. It produces a pronounced degeneration of the spleen (9, 11) and possibly lymph nodes (5), a property that may explain its poor antigenicity in its active form. Little is known of the way in which this interesting toxin acts.

Bordet and coworkers (12) also showed that *B. pertussis* had surface antigens that stimulated agglutinins in animals. They considered all strains of the organism to be a homogeneous group by agglutination, but later Andersen et al. (13) and Eldering et al. (14) showed the presence of at least eight different agglutinogens, of which those designated 1, 2, and 3 by Eldering are major, the others being minor components of the cell. Different combinations of these agglutinogens are found in different strains. Agglutinogen 1, however, is always present in all freshly isolated strains of *B. pertussis*. At least one of these agglutinogens has been purified (15) and found to produce skin reactions in sensitized animals and man (16). Some investigators believe that these surface antigens are very important in the induction of specific protection to whooping cough (17, 18).

Table 1. Biologically Active Substances from *Bordetella pertussis* ᵃ

1. Dermonecrotic toxin (Lienotoxin)
2. Agglutinogens
3. Endotoxin (lipopolysaccharide)
4. Hemagglutinin
5. Pertussigen (histamine-sensitizing factor, lymphocyte-promoting factor, islet-activating factor, mouse-protective antigen, late appearing toxicity factor, heat-labile adjuvant factor)
6. Many different antigenic substances of unknown biological significance when given to animals

ᵃ For references see (9).

A heat-stable toxin was described by Ehrich et al. (19). This toxin resisted heating at 100° C for 1 hour and was later extracted by classical methods used to extract lipopolysaccharides from other Gram-negative bacteria (20–22). This endotoxin has all the typical properties of other endotoxins; it is antigenic, pyrogenic, toxic, and clots the limulus amebocyte lysate in very small concentrations (23). In addition, the endotoxin is a good adjuvant (24); it sensitizes the skin of rabbits to produce a Shwartzman reaction; and given intravenously, it sensitizes Carworth Farms Swiss Webster (CFW) mice to a histamine challenge given 60-120 minutes later but not to challenges given more than 24 hours later (23, 25). Many biological activities of whole cell pertussis vaccine could be due to or complicated by this ever-present endotoxin. Recent studies in our laboratory have shown that endotoxins are good β-adrenergic blocking substances, as determined by their ability to inhibit incorporation of radiolabeled thymidine into DNA of the parotid glands in mice receiving the β-receptor agonist, isoproterenol (R. K. Bergman and J. J. Munoz, 1978 unpublished work). Needless to say, studies on adrenergic receptors would be complicated by endotoxin contamination. The exact mode of action of endotoxins isolated from Gram-negative bacteria other than *B. pertussis* is also largely unknown (26).

A hemagglutinin (HA), first described by Keogh et al. (27, 28), was thought to be responsible for protection of man and mice against *B. pertussis*. However, Masry (29) found that semipurified preparations lacking agglutinogens did not immunize mice to infection with *B. pertussis,* although they were antigenic. More recently Morse and Morse (30) and Sato et al. (31, 49) isolated HA and found that it protected mice from infection. Others (32, 33), however, have found results indicating that the main hemagglutinin does not induce specific immunity. All these workers agree that HA does not sensitize mice to histamine and does not induce lymphocytosis. HA is a protein (30, 31), which can be seen under the electron microscope to consist of 2×40 nm filaments. It is antigenic (34) and attaches to red cells from various animal species (27, 28). The role of this substance in immunity to whooping cough is not known.

Bordetella pertussis cells given to mice produce many changes other than those already mentioned. Most of the biological activities so far observed are listed in Table 2.

Although it may be convenient to attribute them

Table 2. Changes Induced by *Bordetella pertussis* [a]

1. Increased susceptibility to hypovolemic and/or low resistance shock following:
    Histamine
    Serotonin
    Bradykinin
    Endotoxin
    Cold
    X-rays
    Anoxia

2. Adjuvant action on immunological phenomena:
    Autoimmune diseases (EAE, thyroiditis, aspermatogenesis)
    *Production of antibodies of different Ig classes—IgM, IgG
    Production of antibodies of IgE class
    Anaphylaxis (passive and active)
    *Increases delayed hypersensitivity to protein antigens
    Protects mice from specific infection
    *Increases effectiveness of some vaccines
    *Suppresses certain tumors
    *Suppresses delayed hypersensitivity under certain conditions
    *Accelerates some tumor growth

3. Induces various physiological changes:
    *leukocytosis
    lymphocytosis
    depletes lymph organ of lymphocytes
    *hypoglycemia
    hyperinsulinemia
    *hypoalbuminemia
    decreases response to epinephrine
    increases vascular permeability
    inhibits macrophage response to brain injury
    *stimulates ascites and lung edema

4. Lethal or other toxic actions
    *dermonecrotic effects
    *lethal to mice—causes weight loss in low doses
    *lethal to tissue cultures
    *degeneration of spleen
    *enlargement of spleen by detoxified vaccines

[a] For references see (9).
* Activity may be due to substances other than pertussigen.

to different factors, we believe, as others have previously (35), that many of these varied responses are due to a single substance from *B. pertussis*. The first description of this putative substance was made by Parfentjev and Goodline in 1948 (36). In 1955 Maitland et al. (37) referred to it as the histamine-sensitizing factor (HSF). It has since been studied under that name or under the name of lymphocyte-promoting factor (LPF), late appearing toxicity (LAT), mouse-protective antigen (MPA), or more recently as islet-activating protein (IAP). We have proposed the unifying name *pertussigen* for this substance (9). Under this name we include the various factors that have been studied for many years, such as HSF, LPF, heat-labile adjuvant factor, MPA, and the more recently described LAT (38, 39) and IAP (40). We are convinced from our work

and that of others (30–33, 40, 41) that most of the activities indicated in Table 2 (except for those marked with an asterisk) are due to this substance either singly or in combination with other substances of the cell. Admittedly, some of the evidence for this is indirect, but nevertheless it is highly suggestive. Highly purified preparations of pertussigen induce histamine sensitization and lymphocytosis, and they protect mice from infection. Our preparations stimulate antibody production, particularly IgE; accelerate experimental allergic encephalomyelitis (EAE) in rats; and produce hypoglycemia (33). Yajima et al. (40) showed that a highly purified preparation increased the insulin production by the β cells of the pancreas of rats after stimulation by secretagogues. A summary of the activities of the various purified factors made by different workers is given in Table 3. It is clear that in all cases the final products have many of the activities we mentioned above. Some of our purest preparations have had all activities shown in Table 3. We have shown that heating pertussigen at 80° C for 1/2 hour destroys all of these activities (9). The exact chemical composition of pertussigen is not yet fully known, and different workers have obtained different results (Table 4).

One may wonder how a single substance can produce so many apparently different changes in an animal. These changes range from sensitizing an animal to shock to enhancing IgE production or from increasing leukocyte counts to increasing insulin production. It may seem odd at first to suggest that one substance can do all this. However, the realization that one of the actions of pertussigen was to interfere with some functions of epinephrine (47, 56) led to an understanding of how the varied effects can be produced. Epinephrine is a hormone that plays a regulatory role in the physiology of the animal, and any substance interfering with its action can disturb many physiological activities. For example, epinephrine affects permeability to vessels, carbohydrate and fat metabolism, vascular responses to various forms of shock, insulin production, susceptibility to EAE, and maybe even antibody production, as well as other conditions that involve the adrenergic nervous system. Table 5 illustrates how closely a pertussigen-treated mouse compares to an adrenalectomized mouse.

These similarities impressed us early in our investigations of *B. pertussis* (61) and eventually led us to discover that the adrenal medulla and epi-

Table 3. Reported Activities and Properties of Different Factors Described for *Bordetella pertussis*

	Factors				
	HSF (9,42–47)[a]	LPF (30,31,48–54)	IAP (41)	LAT (38,39)	Pertussigen (9,33,55)
Histamine sensitization	+	+	+	+	+
Lymphocytosis	+	+	+	+	+
Insulin production	+		+		+
Loss of weight	+	+		+	+
Protects mice	+	+(?)			+
Accelerates EAE	+				+
Stimulates IgE	+	+			+
Increases anaphylaxis	+				+
Hypoglycemia	+	+(?)	+		+
Blocks epinephrine response	+	+	+(?)		+
Antigenic	+	+	+	+	+(weak)
Inactivated by:					
Formaldehyde	+	+		+	+
Proteolytic enzymes	+(?)			+	(?)
Heat 80° C, ½ h	+	+	+		+
Solubility:					
Water	–	–			–
High salt	+	+	+	+	+
Molecular weight	90,000	87,000	77,000		
Toxicity	>10 μg	4 μg			
Histamine SD$_{50}$ in μg	0.06	0.01	0.02	0.05	0.01

[a] Numbers in parentheses refer to references.

Table 4. Chemical Composition of Various Preparations

	N	P	C	Protein (%)	CHO	Amino Sugar	Lipid	DNA	RNA	Mol. Wt.
Sato and Arai (48)[a]	7.5	1.8	—	46.8	24.5	4.5	17.5	0	0	108,000
Morse and Morse (30)[a]	14.5	—	47.9	100	<1	—	<0.5	—	—	87,000
Lehrer et al. (42)	—	—	—	30.5	2.1	—	57.0	<2	10.4	86,000
Niwa (44)	5.7	2.8	—	—	2.1	<0.4	—	0	0	—
Munoz et al. (33)	10.3	6.8			6.3	0.38	20.2	—	—	—
Yajima et al. (41)[a]	—	—	—	100	—	—	—	—	—	77,000
Arai and Sato (31)[a]		0.26		47.7	25.0		23.9			107,000

[a] Claimed to be pure

Table 5. Similarities Between Adrenalectomized and *Bordetella pertussis*-Treated Mice [a]

Parameter	Adrex	B. pertussis-treated
Histamine shock	+	+
Endotoxin shock	+	+
Anaphylactic shock	+	+
Cold stress	+	+
Susceptibility to EAE	+[b]	+
Effect on sugar and fat metabolism	+	+
Effect on permeability of capillaries	+	+
Adjuvant action	+(?)[c]	+

[a] For references see Munoz and Bergman (9)

[b] Levine et al. (58)

[c] Observed by Murphy and Sturm (59) and Char and Kelley (60) in rabbits

nephrine were important in the sensitivity of mice to histamine (46). Fishel et al. (47) reported that *B. pertussis* blocked various metabolic functions of epinephrine and that the β-adrenergic receptor blocking agents were capable of sensitizing mice to histamine. It is not yet settled whether the β-receptors are the only ones involved, or if they are actually blocked by pertussigen.

The fact that *B. pertussis* blocks some functions of epinephrine, however, may explain the great susceptibility of *B. pertussis*-treated mice to various types of shock. It is more difficult to explain how it produces leukocytosis, increases production of antibodies, or acts in producing protection of mice or

increased susceptibility to EAE. It is known that pertussigen interferes with the recirculation of small lymphocytes, producing a depletion of lymphocytes from the lymph organs and an accumulation in the circulating blood (51–53). How this comes about is not yet known.

The stimulation of antibodies, especially of the IgE type, may be mediated through destruction of suppressor T cells, which normally inhibit the formation of IgE (54), or as Morse et al. (62) have shown, pertussigen may act through its mitogenic effect by increasing the multiplication of plasma cells. We are now investigating some of these problems with Dr. Sadowski in our laboratory. It should be reemphasized that *B. pertussis* endotoxin is an adjuvant and that studies done with whole cell vaccines are difficult to interpret with respect to the contribution made by pertussigen on the one hand and by endotoxin on the other.

In the study of the mechanism by which pertussigen increases the susceptibility of rats to EAE, we have shown that permeability changes in the spinal cord seem to be important in the development of early paralysis (55). Pertussigen, however, does not directly increase the permeability of capillaries (63), although slight changes are observed when normal rats receive pertussigen alone (55). These changes in permeability are insignificant and no clinical or histological EAE is produced. When the encephalitogenic antigen is given concomitantly with pertussigen, paralytic EAE develops by the 7th to 9th day and at this time a marked increase in capillary permeability is observed, mainly in the spinal cord. Why should there be an increased permeability? We know from studies in the mouse that pertussigen blocks some functions of epinephrine (9). One of these functions is to control increases in capillary permeability due to histamine, serotonin, and other vasoactive substances. This activity of epinephrine can easily be shown in the skin of normal and pertussigen-treated mice receiving a small dose of histamine intradermally and the indicator dye Evans Blue intravenously. A much larger area of permeability to the dye is observed in the pertussigen-treated mouse than in the normal animal (63), and epinephrine prevents the spread of the dye. We think pertussigen increases EAE by two main mechanisms. Through its adjuvant action, it increases the sensitization of the animal to the encephalitogen; and it increases the sensitivity of the capillaries to the permeability-increasing effect of histamine, serotonin, or other vasoactive substances released during the reaction of antibody or sensitized cell with the antigen in proximity to the capillary walls. It should be pointed out that rats receiving the encephalitogenic antigen without pertussigen also have a histological picture of EAE, but paralysis occurs rarely. When it does occur it is relatively mild (55). In these animals the permeability changes are minor.

We would like to speculate on the mechanism by which pertussigen may protect mice from the artificial intracerebral infection used in the mouse-protection test. We should start by saying that in our hands fractions of *B. pertussis* that protect mice invariably have histamine-sensitizing activity. However, during fractionation we have seen disproportionate decreases in HSF and protective activities. This has been noticed by other workers as well. Highly purified preparations of pertussigen are protective, but we have never been absolutely sure that small amounts of putative protection-inducing contaminants were not present. Others have shown protection with preparations claimed to be pure. Preparations have been made that were less active in producing sensitization to histamine than in protecting mice (50, 64, 65). This was done by treatment of preparations with formalin.

Pertussigen preparations so far described contain mouse-protective activity. But since cruder preparations, particularly whole cells, are as effective on a weight basis as the highly purified pertussigen, other substances may also be involved in mouse protection. We think these substances may be surface antigens that can react with antibodies and thus make *B. pertussis* cells more susceptible to killing by phagocytic or lytic actions. The most likely substances involved in this protection are agglutinogens, hemagglutinin, and endotoxin. By themselves these substances are not capable of protecting mice by the standard mouse protection test (29, 66–68). Sato has found hemagglutinin effective in protecting mice (see page 51). If pertussigen is combined with these substances, we would expect them to enhance its protective activity. To our knowledge no one has reported the results of such experiments. But, if this actually happens, one can begin to see how pertussigen may act not only as a protective antigen by itself but also as an enhancer of protection by other surface antigens of the cell.

We visualize the situation to be almost identical to the mechanism by which pertussigen increases susceptibility to EAE. Mice are normally rather resistant to *B. pertussis* except when it is given

intracutaneously. In the brain, however, *B. pertussis* is isolated from the normal protecting mechanisms and the bacteria survive and multiply. Eventually the animal is overcome, probably by toxins produced by these cells. When pertussigen alone or in combination with other surface antigen(s) from *B. pertussis* is given 14 days before infection, not only are the antibody and hypersensitivity responses to those antigens accelerated, but the mouse also has a deficient function of epinephrine. Thus slight antibody-antigen (antibody and *B. pertussis* cells in the brain) or sensitized cell-antigen reactions in the proximity of the brain capillaries release vasoactive substances that increase permeability of the blood-brain barrier. The resultant outpouring of more antibodies and cells into the infected brain supplies the essential mechanism for destruction of the bacteria. Pertussigen may be sufficient to protect mice, but it may become more effective when other surface antigens are present. This hypothesis should be subject to experimental proof, and we will try to conduct experiments to support or disprove it.

Before closing, we should mention that although pertussigen seems to interfere with some functions of epinephrine, it is not known exactly how this takes place. Some work, still in its infancy, has been done to localize the effect of pertussigen more precisely. Fishel et al. (47) suggested that the effect was due to a blockade of the β-adrenergic receptors, but various workers have questioned whether pertussigen is a β-receptor blocker (69–71). Some evidence exists that adenylate cyclase may actually be the β-adrenergic receptor, but *B. pertussis* extracts have actually stimulated adenylate cyclase, rather than blocked its action (72). Contradictory results have also been reported on the role that cyclic nucleotides play in the sensitization of mice to histamine (73, 74), and Parker and Morse (70) and Lee (71) have concluded that *B. pertussis* does not act exclusively by blocking the β-adrenergic receptors. We may need to look at other systems affected by epinephrine before we can fully understand the exact site of action of pertussigen at a molecular level.

In conclusion, *B. pertussis* is a cell with many interesting activities produced mainly by the dermonecrotic toxin, the endotoxin, and pertussigen. Of these substances, pertussigen is responsible for many of the immunologic, pharmacologic, and physiologic effects. The action of this substance may be due to its ability to interfere with functions of epinephrine and its adjuvant action. The modes of action of the dermonecrotic toxin and endotoxin are largely unknown.

Future work should be directed toward explaining how pertussigen protects mice, how it produces its adjuvant effect, how it interferes with epinephrine, and what its exact chemical structure is. Such studies would establish whether pertussigen is similar in all strains of *B. pertussis* or is a family of compounds with similar activities in various strains of this bacterium.

In addition, studies should be designed to evaluate the role of agglutinogens and endotoxin in protection from whooping cough and to determine the exact chemical nature of these substances. The dermonecrotic toxin has pronounced effects on the lymph tissue. Its exact chemical composition and its mechanism of action should be determined. The hemagglutinin should be studied further. The genetic control of the production of these active substances should be studied and the culture media components and other conditions of cultivation that affect production of these biologically active substances should be determined more precisely. Finally, and most importantly, we should establish the role these substances play singly and in combination in the prophylaxis of whooping cough.

REFERENCES

1. Kind, L. The altered reactivity of mice after inoculation with *Bordetella pertussis*. Bacteriol. Rev. 22:173–182; 1958.
2. Schweinsberg, H. Zur Antigenstruktur von *Bordetella pertussis*. Behringwerk-Mitt 40:102–141; 1961.
3. Munoz, J. J.; Bergman, R. K. Histamine-sensitizing factors from microbial agents, with special reference to *Bordetella pertussis*. Bacteriol. Rev. 32:103–126; 1968.
4. Pittman, M. Infectious agents and host reactions. Mudd, S., ed. *Bordetella pertussis*—bacterial and host factors in the pathogenesis and prevention of whooping cough. Philadelphia: W. B. Saunders Co., 239–270; 1970.
5. Munoz, J. Microbial toxins. Kadis, S.; Montie, T. C.; Ajl, S. A., eds. Protein toxins from *Bordetella pertussis*; vol. 2A. New York: Academic Press, Inc.; 271–300; 1971.
6. Olson, L. C. Pertussis. Medicine 54:427–469; 1975.
7. Morse, S. I. Biologically active components and properties of *Bordetella pertussis*. Adv. Appl. Microbiol. 20:9–26; 1976.
8. Morse, S. I. Components of *Bordetella pertussis* that modulate the immune response. Schlessinger, D., ed. Microbiology—1977. Washington, D.C.: American Society for Microbiology; 374–377; 1977.
9. Munoz, J. J.; Bergman, R. K. *Bordetella pertussis*—immunological and other biological activities. Immunology Series, vol. 4. New York: Marcel Dekker; 1977:1–235.
10. Bordet, J.; Gengou, O. L'endotoxine coquelucheuse. Ann. Inst. Pasteur 23:415–419; 1909.
11. Iida, T.; Okonogi, T. Lienotoxicity of *Bordetella pertussis* in mice. J. Med. Microbiol. 4:51–61; 1971.

12. Bordet, J; Sleeswyk. Serodiagnostic et variabilite des microbes suivant le milieu de culture. Ann. Inst. Pasteur 24:476–494; 1910.
13. Andersen, E. K. Serological studies on *H. pertussis*, *H. parapertussis* and *H. bronchisepticus*. Acta Pathol. Microbiol. Scand. 33:202–224; 1953.
14. Eldering, G.; Hornbeck, C.; Baker, J. Serological study of *Bordetella pertussis* and related species. J. Bacteriol. 74:133–136; 1957.
15. Onoue, K.; Kitazawa, M.; Yamamura, Y. Chemical studies on cellular components of *Bordetella pertussis*. I. Purification and properties of agglutinogen. J. Bacteriol. 82:648–656; 1961.
16. Flosdorf, E. W.; Felton, H. M.; Bondi, A., Jr.; McGuinness, A. C. Intradermal test for susceptibility to and immunization against whooping cough using agglutinogen from phase I *H. pertussis*. Am. J. Med. Sci. 206:422–425; 1943.
17. Preston, N. W. Potency tests for pertussis vaccines: doubtful value of intracerebral challenge test in mice. J. Pathol. Bacteriol. 91:173–179; 1966.
18. Preston, N. W.; Stanbridge, T. N. Mouse or man? Which are pertussis vaccines to protect? J. Hyg. (Camb.) 76:249–256; 1976.
19. Ehrich, W. E.; Bondi, A., Jr.; Mudd, S.; Flosdorf, E. W. The tolerance of rabbits for the agglutinogen and the toxins of *Hemophilus pertussis*. Am. J. Med. Sci. 204:530–539; 1942.
20. Eldering, G. A study of the antigenic properties of *Hemophilus pertussis* and related organisms. II. Protection tests in mice. Am. J. Hyg. 36:294–302; 1942.
21. MacLennan, A. P. Specific lipopolysaccharides of *Bordetella*. Biochem. J. 74:398–409; 1960.
22. LeDur, A.; Caroff, M.; Chaby, R.; Szabo, L. A novel type of endotoxin structure present in *Bordetella pertussis*. Eur. J. Biochem. 84:579–589; 1978.
23. Bergman, R. K; Milner, K. C.; Munoz, J. J. New test for endotoxin potency based upon histamine sensitization of mice. Infect. Immun. 18:352–355; 1977.
24. Farthing, J. R.; Holt, L. B. Experiments designed to determine the mechanism of the adjuvant activity of Gram-negative organisms upon antibody production. J. Hyg. (Camb.) 60:411–426; 1962.
25. Bergman, R. K.; Munoz, J. J. Increased histamine sensitivity in mice after administration of endotoxins. Infect. Immun. 15:72–77; 1977.
26. Milner, K. C.; Rudbach, J. A.; Ribi, E. General characteristics. Weinbaum, G.; Kadis, S.; Ajl, S. J., eds. Microbial toxins, vol. 4. New York: Academic Press, Inc.; 1–65; 1971.
27. Keogh, E. V.; North, E. A.; Warburton, M. F. Haemagglutinins of *Haemophilus* group. Nature 160:63; 1947.
28. Keogh, E. V.; North, E. A. The haemagglutinin of *Haemophilus pertussis*. I. Haemagglutinin as a protective antigen in experimental murine pertussis. Aust. J. Exp. Biol. Med. Sci. 26:315–322; 1948.
29. Masry, F. L. G. Production, extraction and purification of haemagglutinin of *Haemophilus pertussis*. J. Gen. Microbiol. 7:201–210; 1952.
30. Morse, S. I.; Morse, J. H. Isolation and properties of the leukocytosis- and lymphocytosis-promoting factor of *Bordetella pertussis*. J. Exp. Med. 143:1483–1502; 1976.
31. Arai, H.; Sato, Y. Separation and characterization of two distinct hemagglutinins contained in purified leukocytosis-promoting factor from *Bordetella pertussis*. Biochim. Biophys. Acta 444:765–782; 1976.
32. Nakase, Y.; Doi, M.; Kasuga, T. Hemagglutinating substance of *Bordetella pertussis*. Jpn. J. Med. Sci. Biol. 28:57–59; 1975.
33. Munoz, J. J.; Bergman, R. K.; Cole, R. L.; Ayers, J. C. Purification and activities of pertussigen, a substance from *Bordetella pertussis*. Rosenberg, P., ed. Proceedings of the Fifth International Symposium, San Jose, California, Oxford and New York: Pergamon Press 1015–1029; 1978.
34. Fisher, S. The haemagglutinin of *Haemophilus pertussis*. II. Observations on the structure of the haemagglutinating complex of culture supernatants. Aust. J. Exp. Med. Sci. 28:509–516; 1950.
35. Levine, L.; Pieroni, R. E. A unitarian hypothesis of altered reactivity to stress mediated by *Bordetella pertussis*. Experientia 22:797–798; 1966.
36. Parfentjev, I. A.; Goodline, M. A. Histamine shock in mice sensitized with *Hemophilus pertussis* vaccine. J. Pharmacol. Exp. Ther. 92:411–413; 1948.
37. Maitland, A. B.; Kohn, R.; MacDonald, A. D. The histamine-sensitizing property of *Haemophilus pertussis*. J. Hyg. (Camb.) 53:196–211; 1955.
38. Kurokawa, M.; Iwasa, S.; Ishida, S. On a possible new kind of toxic substance produced by *Bordetella pertussis*. Jpn. J. Med. Sci. Biol. 18:161–165; 1965.
39. Kurokawa, M.; Ishida, S.; Iwasa, S. Asakawa, S.; Kuratsuka, K. Attempt at analysis of toxicity of pertussis vaccine. I. Body weight-decreasing toxicity in mice. Jpn. J. Med. Sci. Biol. 21:137–153; 1968.
40. Yajima, M. Hosoda, K.; Kanbayashi, Y.; Nakamura, T.; Nogimori, K.; Mizushima, Y.; Nakase, Y.; Ui, M. Islets-activating protein (IAP) in *Bordetella pertussis* that potentiates insulin secretory responses of rats. J. Biochem. (Tokyo) 83:295–303; 1978.
41. Yajima, M.; Hosoda, K.; Kanbayashi, Y.; Nakamura, T.; Takahashi, T.; Ui, M. Biological properties of islets-activating protein (IAP) purified from the culture medium of *Bordetella pertussis*. J. Biochem. (Tokyo) 83:305–312; 1978.
42. Lehrer, S. B.; Tan, E. M.; Vaughan, J. H. Extraction and partial purification of the histamine-sensitizing factor of *Bordetella pertussis*. J. Immunol. 113:18–26; 1974.
43. Lehrer, S. B.; Vaughan, J. H.; Tan, E. M. Immunological and biochemical properties of the histamine-sensitizing factor from *Bordetella pertussis*. J. Immunol. 114:34–39; 1975.
44. Lehrer, S. B.; Nakamura, R. M.; Tan, E. M. Histopathological changes in mice treated with extracts of the histamine-sensitizing factor of *Bordetella pertussis*. Int. Arch. Allergy Appl. Immunol. 54:129–142; 1977.
45. Niwa, M. Histamine sensitizing factor of *Bordetella pertussis*. J. Biochem. (Tokyo) 51:222–230; 1962.
46. Bergman, R. K.; Munoz, J. Protection against histamine shock by catecholamines in *Bordetella pertussis*-treated, adrenalectomized or adrenergic blocked mice. Proc. Soc. Exp. Biol. Med. 122:428–433; 1966.
47. Fishel, C. W.; Szentivanyi, A.; Talmage, D. W. Sensitization and desensitization of mice to histamine and serotonin by neurohormones. J. Immunol. 89:8–18; 1962.
48. Sato, Y.; Arai, H. Leucocytosis-promoting factor of *Bordetella pertussis*. I. Purification and characterization. Infect. Immun. 6:899–904; 1972.
49. Sato, Y.; Arai, H.; Suzuki, K. Leukocytosis-promoting factor of *Bordetella pertussis*. II. Biological properties. Infect. Immun. 7:992–999; 1973.
50. Sato, Y.; Arai, H.; Suzuki, K. Leukocytosis-promoting factor of *Bordetella pertussis*. III. Its identity with protective antigen. Infect. Immun. 9:801–870; 1974.
51. Morse, S. I.; Riester, S. K. Studies on the leukocytosis and lymphocytosis induced by *Bordetella pertussis*. I. Radioautographic analysis of the circulating cells in mice

undergoing pertussis-induced hyperleukocytosis. J. Exp. Med. I25:401–408; 1967.

52. Morse, S. I.; Riester, S. K. Studies on the leukocytosis and lymphocytosis induced by *Bordetella pertussis*. II. The effect of pertussis vaccine on the thoracic duct lymph and lymphocytosis of mice. J. Exp. Med. 125: 619–628; 1967.

53. Morse, S. I.; Barron, B. A. Studies on the leukocytosis and lymphocytosis induced by *Bordetella pertussis*. III. The distribution of transfused lymphocytes in pertussis-treated and normal mice. J. Exp. Med. 132:663–672; 1970.

54. Tada, T.; Okumura, K.; Ochiai, T.; Iwasa, S. Effect of lymphocytosis-promoting factor of *Bordetella pertussis* on the immune response. II. Adjuvant effect for the production of reaginic antibody in the rat. Int. Arch. Allergy Appl. Immunol. 43:207–216; 1972.

55. Bergman, R. K.; Munoz, J. J.; Portis, J. L. Vascular permeability changes in the central nervous system of rats with hyperacute experimental allergic encephalomyelitis induced with the aid of a substance from *Bordetella pertussis*. Infect. Immun. 21:627–637; 1978.

56. Bergman, R. K.; Munoz, J. Adrenal medullary hormones and sensitization of mice to histamine by the histamine sensitizing-factor of *Bordetella pertussis*. Nature. 205: 910–911; 1965.

57. Bergman, R. K.; Munoz, J. Protection against histamine shock by catecholamines in *Bordetella pertussis*-treated, adrenalectomized or adrenergic blocked mice. Proc. Soc. Exp. Biol. Med. 122:428–433; 1966.

58. Levine, S.; Wenk, E. J.; Muldoon, T. N.; Cohen, S. G. Enhancement of experimental allergic encephalomyelitis by adrenalectomy. Proc. Soc. Exp. Biol. Med. 111:383–385; 1962.

59. Murphy, J. B.; Sturm, E. The lymphoid tissue and antibody formation. Proc. Soc. Exp. Biol. Med. 66: 303–307; 1947.

60. Char, D. F. B.; Kelley, V. C. Serum antibody and protein studies following adrenalectomy in rabbits. Proc. Soc. Exp. Biol. Med. 109:599–602; 1962.

61. Munoz, J.; Schuchardt, L. F. Sensitivity to histamine of adrenalectomized mice from different strains. J. Allergy Clin. Immunol. 25:125–129; 1954.

62. Morse, J. H.; Kong, A. S.; Lindenbaum, J.; Morse, S. I. The mitogenic effect of the lymphocytosis promoting factor from *Bordetella pertussis* on human lymphocytes. J. Clin. Invest. 60:683–692; 1977.

63. Bergman, R. K.; Munoz, J. J. Effect of *Bordetella pertussis* extracts and vasoactive amines on vascular permeability. J. Allergy Clin. Immunol. 55:378–385; 1975.

64. Pittman, M. Influence of preservatives, of heat, and of irradiation on mouse protective activity and detoxification of pertussis vaccine. J. Immunol. 69:201–216; 1952.

65. Munoz, J.; Hestekin, B. M. Antigens of *Bordetella pertussis*. IV. Effect of heat, merthiolate, and formaldehyde on histamine-sensitizing factor and protective activity of soluble extracts from *Bordetella pertussis*. J. Bacteriol. 91:2175–2179; 1966.

66. Eldering, G.; Holwerda, J.; Baker, J. Mouse-protective properties of *Bordetella pertussis* serotypes in passive tests. J. Bacteriol. 93:1758–1761; 1967.

67. Eldering, G.; Holwerda, J.; Baker, J. *Bordetella pertussis* culture having only species factor 1. J. Bacteriol. 91: 1759–I762; 1966.

68. Schuchardt, L. F.; Munoz, J.; Verway, W. F.; Sagin, J. F. The relationship of agglutinogen to other antigens of *Bordetella pertussis*. J. Immunol. 91:107–111; 1963.

69. Bergman, R. K.; Munoz, J. Effects of epinephrine, norepinephrine and isoproterenol against histamine challenge in *Bordetella pertussis*-treated and β-adrenergic blocked mice. Life Sci. 10:561–568; 1971.

70. Parker, C. W.; Morse, S. 1. The effect of *Bordetella pertussis* on lymphocyte cyclic AMP metabolism. J. Exp. Med. 137:1078–1090; 1973.

71. Lee, T. P. Effect of histamine-sensitizing factor and cortisol on lymphocyte adenyl cyclase responses. J. Allergy Clin. Immunol. 59:79–82; 1977.

72. Cronholm, L. S.; Fishel, C. W. Histamine hypersensitivity of mice indued by 5'AMP. Proc. Soc. Exp. Biol. Med. 127:1178–1180; 1968.

73. Ortez, R. E.; Klein, T. W.; Szentivanyi, A. Impairment of the adenyl cyclase system of mice sensitized bacterially or by beta pharmacological blockade. J. Allergy Clin. Immunol. 45:111;1970.

74. Matsumura, Y.; Vaughan, J. H.; Tan, E. M. The protective effects of nucleotides on the histamine hypersensitivity of *Bordetella pertussis*-treated mice. J. Allergy Clin. Immunol. 54:191–199; 1974.

In Vitro Effects of Lymphocytosis-Promoting Factor on Murine Lymphocytes

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ABSTRACT

Although mitotic activity of mouse lymphocytes is not induced in vivo by doses of lymphocytosis-promoting factor (LPF) that cause lymphocytosis, LPF is a potent T cell mitogen in vitro and at appropriate doses is at least as effective as PHA or Con A. The T cell response requires an accessory non-T, nylon fiber-adherent cell that is not a macrophage. The accessory cell, which itself is not stimulated by LPF, is present in the spleens of adult mice but is difficult to detect in newborn mice. The accessory cell adheres to an anti-Ig column, is lysed by anti-Ig serum plus complement, and recently has been directly shown, by means of a fluorescence activated cell sorter, to be an Ig-bearing cell and therefore a B cell.

Assay by release of ^{51}Cr shows that lymphocytes stimulated in vitro by LPF also exhibit cytotoxicity in vitro. Cytotoxic cells, like the proliferating cells, are T cells. In contrast to other mitogens, additional LPF does not have to be added during the assay to achieve optimal effect. The effector cells are toxic for syngeneic, semisyngeneic, and allogeneic tumor cell targets and for allogeneic and semisyngeneic normal cells, but not for syngeneic normal cells.

INTRODUCTION

The lymphocytosis promoting factor (LPF) isolated from culture supernatant fluids of Phase I *Bordetella pertussis* strain 114 is a basic protein of approximately 73,000 mw (1). It is immunologically homogeneous and migrates as a single band in polyacrylamide gels under nondissociating conditions. When LPF is dissociated with SDS and 2-ME, gel electrophoresis reveals four distinct polypeptides.

Several in vivo effects are produced in experimental animals by *B. pertussis* and its products (reviewed in Refs. 2 and 3). These include heightened antibody response and cell-mediated immune reactions; increased production of reaginic antibody; sensitization to the lethal effects of histamine, serotonin, and a variety of nonspecific stresses; hypoglycemia and unresponsiveness to the hyperglycemic action of epinephrine; hyperleucocytosis with a predominating lymphocytosis; and acute toxicity manifested locally by dermonecrosis and systemically by death of the animals. Although it was thought that these activities were brought about by separate entities, it is now clear that purified LPF is responsible not only for the development of lymphocytosis but also for histamine-sensitization and epinephrine-insensitive hypoglycemia. With respect to the latter it is significant that the islet-activating protein (IAP), described recently by

Yajima et al. (4, 5), which enhances insulin secretion in response to a variety of insulin secretagogues, appears to be identical to LPF. There is also evidence that LPF is the product of *B. pertussis* that enhances reaginic antibody production (6, 7, and S. I. Morse, unpublished observations).

Primary interest in our laboratory over the past several years has been in the mechanism of the lymphocytosis produced in mice by LPF. Many different approaches have shown clearly that the lymphocytosis is caused by redistribution of cells from lymphoid tissues into the circulation. It appears that the redistribution is due to inability of recirculating lymphocytes to leave the circulation and return to lymphoid tissues (8, 9); i.e., the lymphocytes enter the circulation at a normal or elevated rate but are unable to exit at the appropriate sites of their migratory pathway. It is not clear whether emigration from the blood is inhibited by an effect of LPF on the lymphocytes or on the vessels, or whether the effect of LPF is primary or requires a host effector substance. In any case, LPF is not a mitogen for lymphocytes in vivo and the lymphocytosis is not due to the proliferation of new cells (10). The numbers of both circulating T cells and B cells are increased during LPF-induced lymphocytosis in experimental animals (1) and in clinical whooping cough (11).

Although LPF is not a mitogen for lymphocytes in vivo, work in our laboratory showed it is a mitogen for murine T lymphocytes in vitro (12, 13). Subsequently, LPF was shown to be an in vitro mitogen for human T cells (14, 15). Although LPF is clearly a T cell mitogen in the murine system, an accessory cell, adherent to nylon wool, is necessary for the nonadherent T cells to respond. This paper summarizes our findings on the helper cell requirements for T cell proliferation and the identification of the cell. In addition, the generation of cytotoxic T cells by LPF and their activity are described. Detailed experimental results will be presented elsewhere (A. S. Kong and S. I. Morse; and M.-K. Ho, A. S. Kong, and S. I. Morse; manuscripts submitted for publication).

MATERIALS AND METHODS

LPF

LPF was prepared from supernatant fluids of cultures of Phase I strain 114 (formerly strain 3779) by methods described previously (1). Preparations were tested for homogeneity by immunologic and polyacrylamide gel techniques. Biological activity was tested by the capacity to induce leucocytosis. LPF was used as a mitogen at a final concentration of 2 μg/ml.

Animals and Cell Suspensions

Adult female CBA/J mice, obtained from Jackson Laboratories, Bar Harbor, Maine, or bred in this department from Jackson stock were used. Single-cell suspensions were prepared by gently teasing organs in medium and allowing clumps of cells and fibrous tissue to sediment. Bone marrow cells were obtained from femoral marrow plugs expelled by injection of medium into the cavity. All suspensions were washed once in medium, and only those in which 90% or more of the cells were viable by the Trypan Blue dye exclusion test were used.

Thymectomized, B cell reconstituted mice (TxB) were 6-week-old CBA females. After thymectomy they were subjected to lethal X-irradiation and immediately injected with 10^7 liver cells from 14 to 16-day CBA embryos.

Fractionation of Lymphocytes

Spleen cells were separated into nylon wool-adherent B cell-enriched, and nonadherent T cell-enriched populations by the methods of Julius et al. (16) and Handwerger and Schwartz (17).

Separation of cells was performed on discontinuous bovine serum albumin (BSA) gradients (Path-o-cyte 4, Miles Laboratories, Inc., Elkhart, Indiana) consisting of equal volumes of 32, 29, 26, and 23% BSA in a 17 ml cellulose nitrate tube onto which 3 ml of 10% BSA containing 6.0×10^8 lymphocytes was layered. The tubes were centrifuged at 17,500 rpm for 30 minutes at 4° C in an SW 27 rotor.

T cells expressing Thy-1,2 antigen were lysed by Thy-1,2 antiserum (kindly supplied by Dr. M. L. Howe) plus complement (18).

Cell Cultures and ^{3}H-Thymidine Incorporation

The techniques and materials for lymphocyte culture and assay of ^{3}H-thymidine incorporation were those described previously (12). In brief, RPMI-1640 medium (Grand Island Biological Co., Grand Island, New York) supplemented with 5% fetal calf serum (FCS), penicillin, (100 U/ml) and streptomycin, (100 μg/ml) was used. Cell suspensions usually contained 2×10^6 viable cells/ml, and 0.25 ml containing 0.5×10^6 cells was dispensed in triplicate into wells of flat-bottom microtiter plates (model IS–FB–96TC, Linbro Products, New Haven, Connecticut). The plates were incubated in a humid atmosphere of 5% CO_2 and 95% air for 72 hours unless otherwise specified. Eight hours before harvest 2 μCi of ^{3}H-thymidine (SA 5 Ci/mmole) in 0.02 ml was added to each well. Cells were harvested on glass fiber filter papers and washed with saline. After drying, the filters were suspended in scintillation mixture and counted.

Preparation and Assay of Cytotoxic Lymphocytes

One ml of spleen or lymph node cells was added to an equal volume of medium containing 4 μg of LPF and incubated for 3 days. The cells were then washed once and resuspended in fresh medium. 4×10^4 target cells, previously labeled with ^{51}Cr ($Na_2{}^{51}CrO_4$, Amersham Searle, Arlington Heights, Illinois), in 0.2 ml were added to tubes containing 0.25 ml of an appropriate number of LPF-activated cells. After 5 hours at 37° C, 2 ml of medium was added, the tubes were centrifuged, and an aliquot of the supernatant was removed for counting. Percent specific lysis was calculated by the formula:

$$\frac{{}^{51}Cr \text{ release with stimulated lymphocytes} - {}^{51}Cr \text{ release with control cells}}{\text{Maximum } {}^{51}Cr \text{ release} - {}^{51}Cr \text{ release with control cells}} \times 100$$

Maximum ^{51}Cr release was determined by freezing and thawing target cells. Control cells were lymphocytes cultured in the absence of LPF.

RESULTS

Tissue Distribution and Ontogeny of the Accessory Cell

We first studied the ability of cells obtained from various organs to serve as accessory helper cells in the response of T cells to LPF. The responding cells were either T cell-enriched spleen cells that did not adhere to nylon wool or suboptimal numbers of lymph node cells. The proportion of responding cells and accessory cells was varied, but the total cell concentration was maintained at 2×10^6/ml. In addition to nylon wool-adherent spleen cells, helper cells were found in the bone marrow but not in the thymus of adult mice. Indeed, thymocytes suppressed the response of suboptimal numbers of lymph node cells (19).

We had found previously that spleen cells from CBA mice less than 3 weeks old are not stimulated by LPF. This could be due to a deficit in either responsive T cells or helper cells, or to an excess of suppressor cells. Spleen cells from 8 to 18-day-old mice, though themselves unresponsive to LPF, markedly enhanced the response of lymph node cells and nonadherent spleen cells. Splenocytes from mice 4–7 days old exerted only a minimal synergizing effect on nonadherent spleen cells and did not synergize at all with lymph node cells.

Although fetal liver did not contain accessory cells, it contained precursors of the helper cells, since the spleens of T $\times$ B mice contained accessory cells.

Nature and Properties of the Accessory Cell

When adherent spleen cells were centrifuged on discontinuous BSA gradients, accessory cells were localized to the interfaces between the two lightest BSA solutions; 10–23% and 23–26%. The recovery of cells from the gradients was approximately 90%, so there was no false enrichment or loss of accessory cells in any fraction.

One candidate for the nylon wool-adherent accessory cell was a cell of the monocyte-macrophage lineage, but previous experiments had shown that neither peritoneal macrophages nor 2–ME restored the response of nonadherent spleen cells to LPF (13). Moreover, in the present studies we found that the synergistic properties of nylon wool-adherent spleen cells were maintained after cells that had ingested carbonyl-iron were removed or after the cells were passed through Sephadex G–10 columns. Taken together, these observations make it seem highly unlikely that typical macrophages were responsible for helper function.

However, when adherent cells were passed through a column of glass beads to which rabbit anti-mouse Ig was attached (20), the effluent cells did not synergize, whereas the retained cells did. Also, treatment of adherent spleen cells with anti-Ig antisera plus complement resulted in loss of synergistic capacity coincident with the loss of over 95% of cells with surface Ig (sIg$^+$). These results strongly suggest that the synergizing cell was an sIg$^+$ cell and therefore a B cell. Direct confirmation that the helper cell was a B cell was obtained using a fluorescence-activated cell sorter.

Generation of Cytotoxic Lymphocytes by LPF

CBA spleen and lymph node cells cultured in the presence of LPF for 72 hours exerted a cytotoxic effect on P815 DBA mastocytoma cells labeled with ^{51}Cr. The specific lysis by spleen cells at various effector-to-target cell ratios was: 40:1, 85%; 30:1, 80%; 20:1, 68%; 10:1, 41%; and 5:1, 16%. When lymphocytes stimulated by LPF were treated with anti-Thy-1,2 antiserum, cytotoxicity was abolished indicating that the effector cells, like the proliferating cells, were T cells. Cytotoxic effector cells were not readily detectable after spleen cells had been incubated for 48 hours with LPF, whereas incorporation of ^{3}H-thymidine was significant at that time. Nevertheless, proliferation appeared to be necessary for generation of cytotoxic T cells, since no cytotoxic cells were found when mitomycin-C treated cells were cultured with LPF. Moreover, nonadherent cells cultured alone for 72 hours with LPF did not exhibit cytotoxic activity.

There was no evidence of a soluble effector substance; supernatant fluids from spleen cell cultures incubated for 72 hours with LPF had no cytotoxic effect on P815 target cells. LPF itself was not cytotoxic.

Specificity of LPF-Generated Cytotoxic Lymphocytes

Spleen and lymph node cells from CBA (H-2^k) mice were cytotoxic for P815 mastocytoma cells when cultured with LPF. Because the P815 mastocytoma originally arose from DBA/2 (H-2^d) mice, we were interested in determining the immunological specificity of LPF-generated cytotoxic cells

in allogenic, semisyngeneic (strains identical at the major histocompatibility complex, H-2, but with different minor antigens), and syngeneic systems, using both tumor and normal cells as targets. Numerous experiments demonstrated that LPF caused the development of cells cytotoxic for allogeneic and semisyngeneic tumor and normal cells and syngeneic tumor cells. Normal syngeneic cells, however, were not affected.

DISCUSSION

It is clear from the data that the accessory cell required for the proliferation of mouse T lymphocytes in response to LPF possesses surface Ig and is a B cell. This is an unexpected finding, for although T–T cell (22–24) and macrophage-T cell interactions (25, 26) have been reported to be important for the activity of some mouse T cell mitogens, there are no previous reports of a requirement for B–T cell interaction. The mechanism by which B cells "help" the response of T cells to LPF is unknown, but so far there is no evidence that soluble factors are involved. The ontogenic studies on the helper cell indicate that a specific subpopulation of B cells is involved. Although B cells are present in the spleens of week-old mice, no synergy by spleen cells from these animals is found. Studies on other surface markers of the accessory cells, such as complement and Fc receptors, are in progress. Soluble factors do not appear to be responsible for the effect of helper B cells.

Cytotoxic T cells are generated when spleen or lymph node cells are cultured with LPF. It is likely that the cytotoxic cells are a distinct subpopulation of T cells, since these are only demonstrable 24 hours after ^{3}H-thymidine incorporation is manifested significantly. Unlike the situation with cytotoxic T cells generated by PHA or Con A, addition of the mitogen to the cytotoxic assay system itself is not required. Presumably, the addition of PHA or Con A (27, 28) provides an agglutinating "glue" that brings the effector and target cells close together. In the case of LPF-generated killer cells, LPF can be demonstrated on the surface of the cultured lymphocytes, but it is not certain whether the surface LPF subsumes the role of added mitogen or plays no role in the reaction.

It is also clear from these studies that LPF causes generation of cytotoxic T cells that exhibit immunological specificity. They do not cause lysis of syngeneic normal cells but do lyse syngeneic tumor cells as well as allogeneic and semisyngeneic normal

cells and tumor cells. Thus it is possible that tumor-specific surface antigens, antigens of the H-2 complex, and minor histocompatibility antigens are recognized by the LPF-induced effector cells, but it remains to be determined whether a given effector cell has more than one specificity.

REFERENCES

1. Morse, S. I.; Morse, J. H. Isolation and properties of the leukocytosis- and lymphocytosis-promoting factor of *Bordetella pertussis*. J. Exp. Med. 143:1483–1502; 1976.
2. Morse, S. I. Biologically active components and properties of *Bordetella pertussis*. Adv. Appl. Microbiol. 20:9–26; 1976.
3. Munoz, J. J.; Bergman, R. K. *Bordetella pertussis: immunological and other biological activities.* New York: Marcel Dekker; 1977.
4. Yajima, M.; Hosada, K.; Kanbayashi, Y.; Nakamura, T.; Nogimori, K.; Mizushima, Y.; Nakase, Y.; Ui, M. Islets-activating protein (IAP) in *Bordetella pertussis* that potentiates insulin secretory responses of rats. J. Biochem. (Tokyo) 83:295–303; 1978.
5. Yajima, M.; Hosada, K.; Kanbayshi, Y.; Nakamura, T.; Takahashi, I.; Ui, M. Biological properties of islets-activating protein (IAP) purified from the culture medium of *Bordetella pertussis*. J. Biochem. (Tokyo) 83:305–312; 1978.
6. Tada, T. K.; Okumura, T.; Ochiai, T.; Isasa, S. Effect of lymphocytosis-promoting factor of *Bordetella pertussis* on the immune response. II. Adjuvant effect for the production of reaginic antibody in the rat. Int. Arch. Allergy Appl. Immunol. 43:207–216; 1972.
7. Lehrer, S. B.; Tan, E. M.; Vaughan, J. H. Extraction and partial purification of the histamine-sensitizing factor of *Bordetella pertussis*. J. Immunol. 113:18–26; 1974.
8. Morse, S. I.; Barron, B. A. Studies on the leukocytosis and lymphocytosis induced by *Bordetella pertussis*. III. The distribution of transfused lymphocytes in pertussis-treated and normal mice. J. Exp. Med. 132:663-672; 1970.
9. Taub, R. N.; Rosett, W.; Adler, A.; Morse, S. I. Distribution of labeled lymph node cells in mice during the lymphocytosis induced by *Bordetella pertussis*. J. Exp. Med. 136:1581–1593; 1972.
10. Morse, S. I.; Riester, S. K. Studies of the leukocytosis and lymphocytosis induced by *Bordetella pertussis*. I. Radioautographic analysis of the circulating cells in mice

undergoing pertussis-induced hyperleucocytosis. J. Exp. Med. 125:401–408; 1967.

11. Tsukimoto, I.; Lampkis, B. C. Lymphocyte subpopulations in *pertussis*. J. Pediatr. 88:826–828; 1976.

12. Kong, A. S.; Morse, S. I. The in vitro effects of *Bordetella pertussis* lymphocytosis-promoting factor on murine lymphocytes. I. Proliferative response. J. Exp. Med. 145:151–162; 1977.

13. Kong, A. S., Morse, S. I. The in vitro effects of *Bordetella pertussis* lymphocytosis-promoting factor on murine lymphocytes. II. Nature of the responding cells. J. Exp. Med. 145:163–174; 1977.

14. Andersen, V., Hertz, J. B.; Sørensen, S. F.; Baekgaard, P.; Christensen, P. E.; Ramhøj, W.; Hansen, G. A.; Wardlaw, A. C.; Sato, Y. In vitro stimulation of human lymphocytes by *Bordetella pertussis*. Acta Pathol. Microbiol. Scand. Sect. C 85:65–72; 1972.

15. Morse, J. H.; Kong, A. S.; Lindenbaum, J.; Morse, S. I. The mitogenic effect of the lymphocytosis promoting factor from *Bordetella pertussis* on human lymphocytes. J. Clin. Invest. 60:683–692; 1977.

16. Julius, M. H.; Simpson, E.; Herzenberg, L. A. A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol. 3:645–649; 1973.

17. Handwerger, B. S.; Schwartz, R. H. Separation of murine lymphoid cells using nylon wool columns. Transplantation 18:544–548; 1974.

18. Reif, A. E.; Allen. J. M. V. Mouse thymic iso-antigens. Nature 209:521–525; 1966.

19. Ho, M.-K.; Kong, A. S.; Morse, S. I. Influence of non-responding cells on the mitogenic response of murine

20. Wigzell, H. Specific affinity fractionation of lymphocytes using glass or plastic bead columns. Scand. J. Immunol. Suppl. 5 5:23–30; 1976.

21. Howe, M. L.; Cohen, L. Lymphoid cell subpopulations. I. Synergy between lymph node cells and thymocytes in response to alloantigens and mitogens. J. Immunol. 115:1227–1232; 1975.

22. Stent, R. D.; Herzenberg, L. A. The Fc receptor on thymus-derived lymphocytes. II. Mitogen responsiveness of T lymphocytes bearing the Fc receptor. J. Exp. Med. 142:1041–1051; 1975.

23. Frelinger, J. A. Ia-bearing cells promote the concanavalin A mitogenic response of Ia-negative T cells. Eur. J. Immunol. 7:447–450; 1977.

24. Piguet, P.-F.; Dewey, H. K.; Vassalli, P. Synergistic and suppression interactions in the response to phytohemagglutinin. J. Exp. Med. 142:1591–1599; 1975.

25. Mills, G.; Monticone, V.; Paetkau, V. The role of macrophages in thymocyte mitogenesis. J. Immunol. 117:1325–1330; 1976.

26. Haba, S.; Raff, M. C. Accessory cell dependence of lectin-induced proliferation of mouse T lymphocytes. Eur. J. Immunol. 7:451–457; 1977.

27. Stavy, J.; Treves, A. J.; Feldman, M. Effect of concanavalin A on lymphocyte-mediated cytotoxicity. Nature 232:56–58; 1973.

28. Asherson, G. L.; Ferluga, J.; Janossy, G. Non-specific cytotoxicity by T cells activated with plant mitogens in vitro and the requirement for plant agent during the killing reaction. Clin. Exp. Immunol. 15:573–589; 1973.

T cells to the lymphocytosis-promoting factor of *Bordetella pertussis*. Fed. Proc. 36:1323, 1977.

Lymphocytosis Promoting Factor from *Bordetella pertussis:* A Mitogen for Human T Lymphocytes

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ABSTRACT

Lymphocytosis-promoting factor (LPF) was purified from the supernatants of *Bordetella pertussis* cultures and shown to induce proliferation of human T lymphocytes in vitro. This activity was demonstrable in concentrated preparations, after removal of factors toxic to lymphocytes, by a simple acid precipitation technique. Further purification steps were monitored by crossed immunoelectrophoresis.

T lymphocyte colony formation in agar is induced by LPF. These colonies are of clonal origin. In 10 healthy adults, the frequency of LPF-induced T lymphocyte colonies was found to be $5,450 \pm 1,800$ per 10^6 mononuclear blood cells.

Separation of T lymphocytes into FC(γ)-receptor-positive and negative cells demonstrated that the colony forming cells are found mainly in the Fc(γ)-receptor-negative subpopulation, whereas the Fc(γ)-receptor-positive cells suppress colony formation.

INTRODUCTION

It has been shown by others (1) that the interactions of *Bordetella pertussis* and its products with mammalian cell membranes are important for the pathogenesis of pertussis. These interactions are of interest for the study of cell membrane structure and function. Lymphocytosis promoting factor (LPF) of *B. pertussis* induces lymphocytosis by its action on cell membranes. In vitro, it stimulates human lymphocytes and causes their proliferation. Various aspects of the stimulation are documented and discussed in this report.

MATERIALS AND METHODS

Preparations of LPF

A lyophilized Danish vaccine strain 3860/57 was cultured for 48 hours on Bordet-Gengou plates. This culture was seeded to a laboratory fermentor containing 8 liters of modified Cohen and Wheeler medium (2). After 24 hours of growth, when the culture was in the exponential growth phase, it was seeded to a pilot fermentor containing 80 liters of modified Cohen and Wheeler medium. After 24 hours of growth this culture was harvested in the late exponential growth phase (u = 4.5 hours).

To ten liters of this culture 5 N HCl was added until the pH was 3.5. After 3 days at 4° C, the precipitate was extracted repeatedly with phosphate-buffered 1 M NaCl, pH 8. The final volume of approximately 130 ml contained 2,000–3,000 LPF units/ml. The protein concentration, measured by the Lowry method, was approximately 0.5 mg/ml.

The material was further concentrated by precipitation with ammonium sulfate (70% saturation) at pH 8. The precipitate was washed in distilled water and redissolved in 0.1 M Tris-buffer in 0.5 M NaCl, pH 10.

Gel filtration on Sephadex G–150 was performed as described by Morse and Morse (3).

In the initial experiments purified LPF preparations were used. They were kindly donated by Y. Sato, First Department of Bacteriology, National Institute of Health, Tokyo.

Immunoelectrophoresis

Crossed immunoelectrophoresis of LPF preparations was carried out as described by Hertz et al. (4).

Preparation and Analysis of Lymphocytes

Mononuclear cells were obtained from the blood of healthy adults by separation on Ficoll-Isopaque. T lymphocytes were quantitated by rosette formation with sheep erythrocytes (E-rosettes); B lympho-

cytes, by the presence of surface membrane immunoglobulin.[1] Purified T lymphocytes were prepared either by passing the mononuclear cell suspension through Ig-anti-Ig coated columns or by sedimentation of E-rosetting cells through Ficoll-Isopaque. Subpopulations of T lymphocytes were prepared by sedimentation of cells with receptors for the Fc part of the IgG molecule, employing rosetting with ox erythrocytes coated with human IgG.[2]

Culture of Lymphocytes

Stimulation of lymphocytes in liquid culture was quantitated by ^{14}C-thymidine incorporation (5).

T lymphocyte colonies were grown in agar. In the one-step procedure, cells are seeded directly in agar medium in the presence of LPF or phytohemagglutinin (PHA); whereas in the two-step procedure, cells are cultured for 18 hours in liquid medium containing LPF or PHA prior to agar seeding.[3]

Conditioned medium was prepared from supernatants of cultures of blood mononuclear cells adherent to plastic dishes and added before seeding to cell suspensions depleted of monocytes, since these cells or factors derived from them are necessary for T lymphocyte colony formation (6).

RESULTS

The supernatants of liquid B. pertussis cultures contain factors that are toxic to lymphocytes (5). These factors can be removed by the simple acid precipitation technique described in Materials and Methods. The preparation obtained contains many antigens (Fig. 1, top). Further purification is obtained by precipitation with ammonium sulfate followed by gel filtration on Sephadex G–150. In fractions inducing lymphocyte stimulation, only one precipitate is visible in crossed immunoelectrophoresis (Fig. 1, bottom).

Purified preparations of LPF induce a striking lymphocyte proliferation in vitro (Fig. 2). By fractionation of human blood lymphocytes, it has been shown that the subpopulations responding to stim-

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[1] Claësson, M. H.; Andersen, V.; Hansen, G. S. Colony formation by subpopulations of human T lymphocytes: I. Effects of phytohemagglutinin lymphocytosis promoting factor from *Bordetella pertussis*. Clin. Exp. Immunol. In press, 1978.

[2] Hansen, G. S. Human leucocyte responses in vitro. V. Transformation of different mononuclear cell suspensions prepared by two-step E- and EA-rosette sedimentation. In preparation.

[3] Claësson, et al., op. cit.

Figure 1. Crossed immunoelectrophoresis of preparations o B. pertussis culture supernatants, containing lymphocytosis promoting and lymphocyte stimulating activity. Top: After acid precipitation. Bottom: Following gel filtration on Sephadex G–150. First dimension: Anode to the right. Second dimension: Anode at the top. Rabbit antiserum was raised against the acid precipitable fraction.
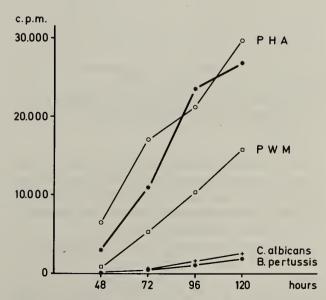

Figure 2. ^{14}C-thymidine incorporation by lymphocytes stimulated with purified LPF (heavy line). For comparison, the responses to phytohemagglutinin (PHA), pokeweed mitogen (PWM), *Candida albicans* extract, and heat-killed B. pertussis are given.

ulation by LPF are those containing T lymphocytes.[4]

After stimulation for 24 hours by LPF in liquid cultures, lymphocytes transferred to semisolid agar containing LPF form colonies. These colonies are the result of clonal growth of T lymphocytes, as shown by the ability of colony cells to form rosettes with sheep erythrocytes.[5] The mean frequency of LPF-induced T lymphocyte colonies in 10 adult donors was 5,450 ± 1,800 per 10^6 mononuclear blood cells (Fig. 3). No colonies were formed when lymphocytes were plated directly in agar containing LPF.
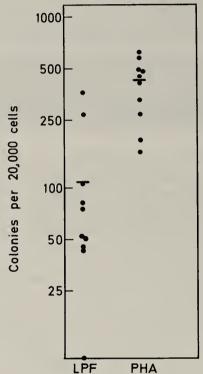
Figure 3. Frequency of LPF-induced T lymphocyte colonies in cultures of mononuclear blood cells from 10 healthy adults. Results of the one-step PHA method are shown for comparison.

If the mononuclear cells prior to culture were passed through a column coated with anti-immunoglobulin, the capacity to form colonies in response to LPF almost disappeared, whereas the response to PHA seemed enhanced (Table 1).

To further characterize the cell population re-

Table 1. T Lymphocyte Colony Formation by Normal and Anti-Immunoglobulin Column Passed Mononuclear Blood Cells

	T Lymphocyte Colonies per 10^4 Cells	
	Before column passage	After column passage
Stimulation by LPF (average ± SEM of four exp)	34 ± 12	3 ± 1.8
Stimulation by PHA (average ± SEM of six exp)	201 ± 32	260 ± 51

sponsible for colony formation, T lymphocytes were separated into $Fc(\gamma)$-receptor-positive and -negative cells. The results of these experiments are summarized in Table 2. Colony-forming cells were found mainly in the $Fc(\gamma)$-receptor-negative population. Cocultivation of $Fc(\gamma)$-receptor-positive and -negative T lymphocytes resulted in more than 50% reduction in the number of colonies.[6]

DISCUSSION

The proliferative response of human blood lymphocytes to heat-killed *B. pertussis* in vitro is largely due to nonspecific stimulation (5). One explanation of this might be cross-reactivity with antigens of other microorganisms, as has been demonstrated by crossed immunoelectrophoresis (7). Another possibility is the presence of polyclonal activators. LPF has been shown to be a potent T lymphocyte mitogen in mice (8) and in humans (5, 9). Our findings that the cells of LPF-induced lymphocyte colonies form rosettes with sheep erythrocytes, and that no immunoglobulin is demonstrable in the cell membrane, are evidence that these colonies are composed of T lymphocytes.

T lymphocyte colonies have previously been induced by PHA only. For growth both LPF- and PHA-induced colonies are dependent on monocytes or on soluble factors released by monocytes. The observation that cells passed through a column coated with anti-immunoglobulin formed large numbers of colonies in response to PHA but very few in response to LPF indicates that cells that can be stimulated by LPF to form colonies or accessory cells necessary for colony formation are retained in the column. This strongly suggests that LPF-induced colonies arise from another subpopulation of T lymphocytes than do PHA-induced colonies.

[4] Andersen, V.; Hertz, J. B.; Nicolaisen, E. M.; Hansen, G. S.; Hansen, G. A., Strøbaek, S.; Christensen, P. E. Stimulation of human lymphocytes by *Bordetella pertussis* and its lymphocytosis promoting factor. Acta Pathol. Microbiol. Scand. In press, 1978.

[5] Claësson, et al., op. cit.

[6] Claësson, et al., op cit.

Table 2. Colony Formation by Fc(γ)-Receptor-Negative (Fc-neg) and Fc(γ)-Receptor-Positive (Fc-pos) T Lymphocytes

	Colonies per 10^4 T Lymphocytes			Mixing Experiments
	Unseparated	Fc-neg	Fc-pos	10^4 Fc-neg + 10^4 Fc-pos
Stimulation by LPF (average ± SEM of three exp)	102 ± 34	270 ± 71	58 ± 46	112 ± 11
Stimulation by PHA (average ± SEM of three exp)	213 ± 73	303 ± 130	56 ± 35	137 ± 98

When T lymphocytes are separated according to their ability to form rosettes with IgG-coated ox erythrocytes, two subpopulations are obtained. The cells capable of forming colonies mainly belong to the Fc(γ)-receptor-negative subpopulation. When equal numbers of Fc(γ)-receptor-positive and -negative T lymphocytes were cocultured, both LPF- and PHA-induced colonies were markedly reduced in numbers. It thus seems that Fc(γ)-receptor-positive T lymphocytes, probably after activation by mitogen, secrete one or more diffusible factor(s) that suppress colony formation by Fc(γ)-receptor-negative T lymphocytes.

In conclusion, analysis of the consequences of interactions between LPF and cell membranes may not only explain the lymphocytosis and possibly other findings in pertussis patients, but may also yield important information about lymphocyte function.

ACKNOWLEDGMENT

REFERENCES

1. Muse, K. E.; Findley, D.; Allen, L.; Collier, A. M. In vitro model of *Bordetella pertussis* infection: pathogenic and microbicidal interactions. This symposium.
2. Lane, A. G. Use of glutamic acid to supplement fluid medium for cultivation of *Bordetella pertussis*. Appl. Microbiol. 19:512–520; 1970.
3. Morse, S. I.; Morse, J. H. Isolation and properties of the leukocytosis- and lymphocytosis-promoting factor of *Bordetella pertussis*. J. Exp. Med. 143:1483–1502; 1976.
4. Hertz, J. B.; Høiby, N.; Andersen, V.; Wardlaw, A. C.; Parton, R.; Baek, L.; Hansen, G. A. Crossed immuno-electrophoretic analysis of *Bordetella pertussis* antigens and of corresponding antibodies. This symposium.
5. Andersen, V.; Hertz, J. B.; Sørensen, S. F.; Baekgaard, P.; Christensen, P. E.; Ramhøj, W.; Hansen, G. A.; Wardlaw, A. C.; Sato, Y. In vitro stimulation of human lymphocytes by *Bordetella pertussis*. Acta Pathol. Microbiol. Scand. Sect. C 85, 65–72; 1977.
6. Claësson, M. H.; Whittingham, S.; Rodger, M. B.; Burgess, A. W. Colony growth of human T lymphocytes in agar: effect of a soluble factor from adherent cells. Eur. J. Immunol. 7:608–611; 1977.
7. Høiby, N.; Hertz, J. B.; Andersen, V. Cross-reactions between *Bordetella pertussis* and twenty-eight other bacterial species. Acta Pathol. Microbiol. Scand. Sect. B 84:395–400; 1976.
8. Kong, A. S.; Morse, S. I. The in vitro effects of *Bordetella pertussis* lymphocytosis-promoting factor on murine lymphocytes. I. Proliferative response. J. Exp. Med. 145:151–162; 1977.
9. Morse, J. H.; Kong, A. S.; Lindenbaum, J.; Morse, S. I. The mitogenic effect of the lymphocytosis promoting factor from *Bordetella pertussis* on human lymphocytes. J. Clin. Invest. 60:683–689; 1977.

Investigation of the Biological Activities of the Histamine Sensitizing Factor of *Bordetella pertussis*

S. B. Lehrer

ABSTRACT

Purification of histamine sensitizing factor (HSF) from *Bordetella pertussis* cells by extraction with urea-sodium chloride solvent, gel filtration column chromatography, and ion exchange chromatography resulted in a 320-fold increase in specific HSF activity. The purified fraction was also a potent inducer of leukocytosis and adjuvant for reaginic antibody, suggesting that these diverse biological activities are caused by the same substance. Immunoelectrophoresis and polyacrylamide gel electrophoresis of the purified fraction demonstrated the presence of several components. Therefore fractionation was continued with a carboxymethyl Sephadex column. Again, histamine-sensitizing activity, leukocytosis-promoting activity, and adjuvant activity for reaginic antibody were all contained within the same column fraction, although polyacrylamide gel electrophoretic analysis of this fraction demonstrated the presence of at least three distinct components.

Partially purified HSF was used to determine the time necessary for manifestation of maximal histamine sensitization. Although soluble preparations were used, maximal activity was detected 3 to 4 days after sensitization. To determine if this latent period is due to uptake of the factor, antiserum was injected at different time intervals after HSF treatment to determine if HSF could still react with it as detected by neutralization. The results show that although most of the HSF could be neutralized within 4 hours after treatment, little was neutralized after 24 hours. This suggests that HSF was no longer available to react with antibody and may bind to receptor sites within the first 24 hours after treatment.

INTRODUCTION

The histamine sensitizing factor (HSF) of *Bordetella pertussis* can make mice or rats unusually sensitive to the lethal effects of histamine (1,2). Partially purified extracts of HSF produce profound physiologic changes in test animals, including leukocytosis (2–6) and enhanced production of reaginic and hemagglutinating antibodies (1,2,4,5, 7–9). Since some of these physiologic changes resemble aspects of atopic diseases in man (10), elucidation of mechanisms causing the biological effects of HSF might contribute further understanding of human atopic disease.

There have been many attempts to isolate and characterize HSF in order to analyze structural features contributing to biological activities. The present study describes further isolation of HSF and characterization of its uptake in vitro through use of specific antiserum.

MATERIALS AND METHODS

Biological Assays

Histamine-sensitizing activity was quantitated by the 50% sensitizing dose (SD_{50}) determination. Mice in groups of five were injected intravenously (i.v.) with 0.2 ml test material in phosphate-buffered saline (PBS). After an optimal latent period (3 to 4 days unless otherwise indicated), mice were challenged with intraperitoneal (i.p.) injection containing 1 mg of histamine base. Deaths were recorded for the next hour, and the SD_{50} was determined mathematically from these results by the method of Reed and Muench (11). The total number of SD_{50} in a sample was estimated by dividing test material weight or volume by its SD_{50}.

Lymphocytosis-promoting activity was estimated in groups of five mice that had been i.v. injected with 0.2 ml test material. Three days after injection, animals were bled from the orbital plexus and the number of WBC/mm^3 determined.

To estimate adjuvant activity, mice in groups of five were each injected i.v. with 0.2 ml test material and i.p. with 100 μg ovalbumin (OA) in 0.5 ml PBS. Animals were bled 4 weeks after immunization. The reaginic antibody response was determined by the passive cutaneous anaphylaxis (PCA) reaction of Ovary (12). Hemagglutinating (PHA) response was determined by the microtiter tech-

nique using sheep red blood cells coated with ovalbumin by the method of Onkelinx et al. (13). The details of all assays are described elsewhere (5).

HSF Extract

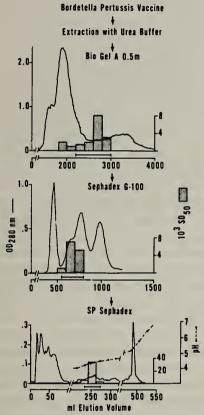The extraction procedure (Fig. 1), has been reported in detail elsewhere (4). *Bordetella per-*

Figure 1. Extraction and partial purification of HSF from *B. pertussis*. HSF was extracted from bacterial cells with urea-sodium chloride buffer. The extract was purified by gel filtration on Bio Gel A 0.5 M and Sephadex G–100 and then by ion exchange chromatography on Sulphopropyl Sephadex C–50. Column fractions were analyzed for ultraviolet absorbing material at 280 nm and for biological activities, which are indicated by the cross-hatched areas.

tussis (donated by Dr. G. R. Anderson, Department of Public Health, Lansing, Michigan) was centrifuged (10,000 × g) and the pelleted bacteria lyophilized and stored under dessication. Bacterial cells were extracted with a solution of 4 M urea and 1 M NaCl buffered with 0.1 M sodium phosphate (urea buffer) at pH 6. The *Bordetella pertussis* extract was purified by gel filtration (Bio Gel A 0.5 M and Sephadex G–100) and ion exchange chromatography (Sulphopropyl Sephadex C–50 column, 4). Histamine-sensitizing activity, leukocytosis-promoting activity, and adjuvant ac-

tivity were all induced by the same column fraction (called SP–HSF).

HSF was purified further by ion exchange chromatography on a 0.8 × 21 cm carboxymethyl Sephadex column (Pharmacia, Piscataway, N.J.). SP–HSF was equilibrated with 4 M urea in 0.05 M citrate, pH 5.0, and applied to the CM-Sephadex column in the same buffer. After eluting at least 50 ml of the same buffer, a continuous linear salt gradient was begun with 40 ml 4 M urea–0.05 M citrate, pH 5.0, and 40 ml 4 M urea–1 M NaCl 0.05 M citrate, pH 5.0. Fractions with biological activity were pooled, concentrated, and centrifuged (105,000 × g). The resulting extract was designated CM–HSF.

Neutralization

Rabbit antisera to HSF were prepared by injecting 40 μg (or an equivalent amount) of HSF into the rabbit's lymph node, followed by injection of 100 μg subcutaneously and in the footpads 27 days later (15). Rabbits were bled 7 to 10 days after the last injection and the antisera were stored at −20° C. To test antisera by neutralization assay for ability to react with HSF, equal amounts of G–100–HSF (diluted 1:10 with PBS) and antisera or normal rabbit serum (NRS) were mixed and incubated for 3 hours at 24° C. The mixture was then centrifuged and the supernatant tested for histamine-sensitizing activity as described. Antiserum was also used for passive neutralization of HSF. Eight-week-old female mice were injected i.v. with 0.2 ml heat-inactivated (56° C, 30 minutes) antiserum or NRS. At different time intervals prior to injection of antiserum, groups of five mice were injected i.v. with G–100–HSF as described previously, to determine if the biologic activity of HSF was affected by passively administered antiserum.

Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoresis was performed as described by Reisfeld and Small (17), with the following modifications. All PAGE gels were 6% polyacrylamide in 4 M urea–0.9 M acid buffer, pH 3.2. Gels were run at 2.5 mA/gel for 4 hours. Gels were gently removed from columns, stained with Coomassie Blue, and destained overnight with a solution of 20% methanol, 5% acetic acid, and 3% glycerin.

To obtain components fractioned by PAGE, six gels were loaded with 50 μg CM-HSF and run as described. After completion of the electro-

phoresis, one gel was stained while the others were immediately frozen on a dry-ice slab and stored at −20° C. The location of bands within the unstained gels was estimated from RF values for each band in the stained gel. A segment (± 3 mm) containing each band was removed, cut into small pieces and eluted for 22 hours at 4° C with 4 ml of 4 M urea–1 M sodium chloride–0.01 M phosphate buffer, pH 5. Each extract was centrifuged to obtain the supernatants.

Immunoelectrophoresis (IEP)

IEP was performed according to the procedure of Grabar and Williams (16) in 0.5% agarose (Marine Colloids, Inc., Rockland, Maine) in 0.05 M barbital buffer, pH 8.2. HSF extracts were added to the wells and electrophoresed at 40 mA/slide, 4° C for 7 hours, after which antisera were added to the trough. Precipitin lines were developed by placing the gels in a moisture chamber at 4° C for 48 hours.

RESULTS

Purification of HSF

The recovery and yield of biological activities of HSF extracted from *Bordetella pertussis* are summarized in Table 1. The amount of material recovered in the purest fraction, SP–HSF, accounted for only 0.0085% of the original starting material. This fraction has an enhanced histamine-sensitizing activity, SD_{50} of 0.055 μg, as compared to an SD_{50} of 18 μg of the bacterial vaccine. This represents a more than 320-fold increase in specific activity. Recovery of activity as assessed by the percent of total SD_{50}s isolated in the purified material was 2.7%, indicating that much activity was lost or destroyed during purification.

Purified fractions were potent inducers of leukocytosis. The most active leukocytosis-promoting fraction was SP–HSF. It also was a potent adjuvant for reaginic and hemagglutinating antibodies. Although all fractions exhibited some adjuvant activity, those with enhanced histamine-sensitizing and leukocytosis-promoting activities clearly contained a greater amount of adjuvant activity for both reaginic and hemagglutinating antibodies.

Polyacrylamide gel electrophoresis of three different pertussis extracts, S3, G–100–HSF, SP–HSF, containing comparable amounts of biological activity, demonstrated that purification had been achieved (Fig. 2). The crudest extract, S3, contained many components that were removed by purification. Nevertheless, SP–HSF still contained many bands. Lack of complete purity was also indicated by immunoelectrophoresis of SP–HSF, in which several different antigens were demonstrated (Fig. 3). Therefore further purification was performed with a carboxymethyl Sephadex column.

Carboxymethyl Sephadex column fractions were analyzed for ultraviolet (UV) absorption (280 nm) and biological activity. The linear gradient eluted several peaks of UV absorbing material (Fig. 4). Maximal histamine-sensitizing activity, leukocytosis-promoting activity, and adjuvant activities were all in the same fractions (descending shoulder of the first peak). Fractions with these activities were pooled, concentrated, and analyzed by PAGE (Fig. 2). Despite purification, CM–HSF still contained several electrophoretically distinct components. Thus HSF was not isolated in completely pure form.

Attempts were made to identify HSF after PAGE of CM–HSF by eluting the three major bands and investigating their histamine-sensitizing activity and

Table 1. Recovery and Yield of HSF Extracted from *Bordetella pertussis*

Bacterial Substance Tested	% Weight Recovered	Histamine Sensitizing Activity		Leukocytosis Promoting Activity 10^3 WBC/mm³	Adjuvant Activity			
					PCA Titer		PHA Titer	
		SD_{50} (μg)	% SD_{50}		5μg	0.05μg	5μg	0.05μg
Bacterial vaccine	100.0	18.00	100.0	NT[a]	NT	NT	NT	NT
Bacterial pellet	32.7	5.94	70.0	19.0[b]	8[c]	<5	640	<20
Extract	6.6	4.30	28.0	17.6	15	<5	1280	<20
G–100–HSF	0.012	0.724	4.7	22.0	80	<5	640	20
SP–HSF	0.0085	0.055	2.7	57.5	80	8	640	320

[a] Not tested
[b] Mean WBC/mm³ of five mice 3 days after i.v. injection of 10 μg test material
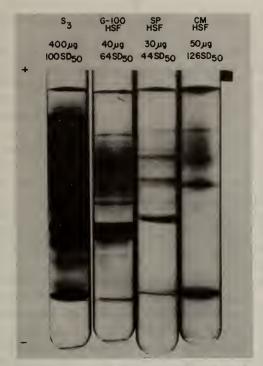[c] Geometric mean of reciprocal maximal antibody titer from three determinations

Figure 2. Polyacrylamide gel electrophoretic analysis of pertussis extracts. Different amounts (30–400 μg) of pertussis extracts containing 44 to 126 SD$_{50}$ in sucrose were gently layered on the top of each gel. All PAGE gels were 6% in 4 M urea-0.9 acetic acid buffer (pH 3.2). Gels were run (2.5 mA/gel) for 4 hours and stained with Coomassie Blue.

Figure 3. Immunoelectrophoretic analysis of pertussis extracts. SP–HSF, G–100–HSF, or S3 was added to the center wells and electrophoresed at 40 mA per slide, 4° C for 7 hours. Antisera were added to the troughs and precipitin lines developed by storage at 4° C in a moisture chamber for 48 hours.

antigenicity (as determined by production of antisera capable of neutralizing histamine-sensitizing activity). Unfortunately, no residual histamine-sensitizing activity was detected in any of the bands isolated. However, some neutralizing activity was present in antiserum to band 1, although it was far less than that of antiserum to CM–HSF or SP–HSF (Table 2).

HSF Uptake in vivo

The in vivo reactivity of antisera with HSF was determined as a function of time. Antiserum to SP–HSF was used in these studies, since other antisera to more purified fractions were not as effective (Table 2). To test the in vivo reactivity of antisera with HSF, different groups of mice were treated with antisera or NRS 4, 8 or 24 hours after injection of HSF. Four days after HSF treatment, the animals were bled and challenged with histamine, and the percent of histamine-sensitizing activity neutralized was calculated. A problem in these studies was that NRS could nonspecifically neutralize histamine-sensitizing activity. Therefore all results were expressed as a percentage of NRS-treated HSF mice. Antisera administered 4 hours

after HSF treatment neutralized most of the activity (Table 3), but as the interval between HSF and antisera injections increased the percent activity neutralized was reduced. By 24 hours after injection of HSF, antisera did not neutralize any more histamine-sensitizing activity than antisera from mice treated with NRS. These results suggest that by 24
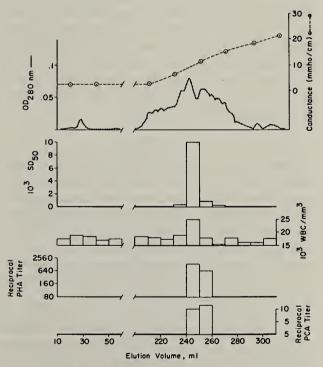

Figure 4. Ion exchange column chromatography of SP–HSF on CM-Sephadex column. SP–HSF in 4 M urea-0.05 M citrate, pH 5.0 was applied to the CM–Sephadex column in the same buffer, and 50 ml of this buffer eluted. Then a continuous linear salt gradient prepared with 40 ml 4 M urea-0.05 citrate, pH 5.0, and 40 ml 4 M urea-1 M NaCl-0.05 M citrate pH 5 was begun. Fractions were analyzed for salt concentration by conductivity (mmho/cm), for protein by U.V. absorption at 280 nm, and for biological activities.

Table 2. Neutralization of HSF by Different Antisera

Antisera Tested	SD$_{50}$	
	Total	% Neutralized [a]
NRS	19,126	0
Anti–SP–HSF	422	98
Anti–CM–HSF	8,032	58
Anti–Band 1	11,064	42
Anti–Band 2	19,126	0
Anti–Band 3	19,126	0

[a] % histamine sensitizing activity neutralized =

$$100\% \quad (1 - \frac{SD_{50}\ Ab}{SD_{50}\ NRS})$$

hours after treatment, HSF is no longer available to react with antisera. Presumably it has bound to receptor sites.

To determine the relationship of HSF uptake to biological activity, groups of five mice injected with different concentrations of HSF were challenged with histamine at different times after injection (Table 4). In mice treated with the greatest dose of HSF, histamine-sensitizing activity was detected by 4 to 8 hours after treatment. Mice treated with smaller doses of HSF did not demonstrate histamine sensitivity until later. If the total number of SD$_{50}$s/ml are calculated, it appears that maximal activity occurs approximately 96 hours after treatment and persists for a long time.

DISCUSSION

HSF is an especially intriguing microbial substance because it appears that this single molecular species may elicit such diverse and seemingly unrelated biological activities as histamine sensitivity, leukocytosis, and adjuvant activities. Understanding the mechanism(s) by which these biological activities are elicited would significantly enhance our understanding of the effects of other microbial substances in human disease.

The object in this and other studies was to isolate HSF in completely purified form and investigate aspects of its molecular structure that might contribute to its unusual biological activities. Unfortunately, the purest HSF fraction isolated still demonstrated several components in polyacrylamide gel electrophoresis.

Another problem encountered was that the stability of the HSF molecule decreased with purification. The isolation of HSF may yet be successful, but the problem of instability must first be over-

come. Variations in buffers and pH may enhance the stability of purified HSF.

Additional studies of HSF were done using rabbit antisera. HSF has been shown to be antigenic, since antisera to it can neutralize its activity. Unfortunately, antisera to the purest fractions isolated, such as CM–HSF or PAGE bands, did not demonstrate much neutralizing activity. Perhaps this is because antigenic configurations may be similar to the structural features that elicit biological activity, and when activity is destroyed the antigenic configurations that elicit antibody production are also destroyed. The purest fractions are not only less stable, they also have been inactivated during the immunization process and have lost their antigenicity.

Further studies were directed at investigating HSF reactivity in vivo. The results demonstrated that high doses of HSF can sensitize mice within 4 to 8 hours after treatment, whereas low doses take a much longer time. Thus maximal biological activity, calculated as total SD$_{50}$, was not demonstrated until 72 to 96 hours after treatment.

We do not know why maximal activity of HSF was detected within several hours, whereas minimal activity was not detected until several days after treatment. It may be that maximal doses of HSF (which contain a greater number of HSF molecules) merely increase the possibility of the HSF molecules attaching to in vivo receptor sites and that minimal doses containing fewer molecules need more time to increase the chance that an HSF molecule will encounter its receptor. If this is true, unbound HSF should still react with antisera within the time interval prior to manifestation of biological activity. This was not the case. By 24 hours after injection (a period in which only a small amount of sensitization has occurred), no histamine-sensitizing activity could be neutralized, which suggests that HSF was no longer available to react with antisera.

Table 3. Neutralization of Biological Activity at Different Time Intervals After Treatment

Time after injection (hours)	SD$_{50}$		% SD$_{50}$ Neutralized [a]
	NRS	Ab	
4	16,300	1,290	91
8	6,400	1,600	75
24	3,170	3,170	0

[a] $100\% (1 - \frac{Ab\ (HSF)}{NRS\ (HSF)})$

Table 4. Histamine Sensitizing Activity of HSF at Different Time Intervals After Treatment

Time after Injection (hours)	Dilution of Sample					
	20[a]	80	320	1280	5120	SD_{50}/ml
1	0/5 [b]	0/5	NT	NT	NT	0
4	2/5	0/5	0/5	NT	NT	10
8	4/5	2/5	0/5	NT	NT	295
16	4/5	3/5	0/5	0/5	NT	400
24	4/5	3/5	1/5	0/5	0/5	499
48	5/5	5/5	1/5	0/5	0/5	951
72	5/5	4/5	3/5	2/5	0/5	2539
96	5/5	5/5	5/5	3/5	1/5	9807
168	5/5	5/5	4/5	1/5	0/5	3200

[a] This dilution contains 146 μg G–100–HSF.

[b] Survivors/total tested

The delayed reactivity of HSR will have to be explained by mechanisms other than uptake. This interpretation excludes any change in HSF antigenicity in vivo, which, if it occurs, could be a source of error in analysis of these studies.

Rather than a simple binding of HSF to an in vivo receptor, it is possible that once HSF has been taken up secondary events must take place before sensitization can occur. However, if this were the case there should be a similar delay in mice treated with high and low doses. Clearly there is not. A problem with these studies is that NRS treatment itself can reduce total histamine-sensitizing activity, and the closer the NRS injection is to the histamine challenge, the greater the reduction. Thus, although all our results are expressed as a percentage of the NRS control treated mice, the control is not a passive reaction and may alter results somewhat.

In summary, HSF has been purified considerably, but the purest HSF fraction isolated still contains several components. Present studies are directed at determining in vivo uptake of HSF and its relation to biological activity. This approach to HSF analysis should provide meaningful information about HSF and its biological activities.

ACKNOWLEDGMENTS

REFERENCES

1. Munoz, J.; Bergman, R. K. Histamine sensitizing factors from microbial agents with special reference to *Bordetella pertussis*. Bacteriol. Rev. 32:103–126; 1968.

2. Munoz, J. J.; Bergman, R. K. *Bordetella pertussis*: immunological and other biological activities, New York and Basel: Marcel Dekker, Inc.; 1977.

3. Morse, S. I. Studies on the lymphocytes induced in mice by *Bordetella pertussis*. J. Exp. Med. 121:49–68; 1965.

4. Lehrer, S. B.; Tan, E. M.; Vaughan, J. H. Extraction and partial purification of the histamine sensitizing factor of *Bordetella pertussis*. J. Immunol. 113:18–26; 1974.

5. Lehrer, S. B.; Vaughan, J. H.; Tan, E. M. Immunological and biochemical properties of the histamine sensitizing factor of *Bordetella pertussis*. J. Immunol. 114:34–39; 1975.

6. Morse, S. I.; Morse, J. H. Isolation and properties of the leukocytosis and lymphocytosis promoting factor of *Bordetella pertussis*. J. Exp. Med. 143:1483–1502; 1976.

7. Clausen, C. R.; Munoz, J.; Bergman, R. K. A reaginic type of antibody stimulated by extracts of *Bordetella pertussis*. J. Immunol. 103:768–777; 1969.

8. Pieroni, R. E.; Levine, L. Further studies on the adjuvant principle of pertussis vaccine for the mouse. Nature 213:1015–1017; 1967.

9. Lehrer, S. B.; Vaughan, J. H.; Tan, E. M. Adjuvant activity of the histamine sensitizing factor of *Bordetella pertussis* in different strains of mice. Int. Arch. Allergy Appl. Immunol. 49:796; 1975.

10. Szentivani, A. The beta adrenergic theory of the atopic abnormality in bronchial asthma. J. Allergy 42:203–232; 1968.

11. Reed, L. J.; Muench, H. A simple method of estimating 50% endpoints. Am. J. Hyg. 27:493–497; 1938.

12. Ovary, Z. Passive cutaneous anaphylaxis. Ackroyd, J. F., ed. Immunological methods. Oxford: Blackwell; 1964: 259–283.

13. Onkelinx, E.; Meuldermans, W.; Joniu, M.; Loulie, R. Glutaraldehyde as a coupling reagent in passive hemagglutination. Immunology 16:35–43; 1969.

14. Lehrer, S. B.; Nakamura, R. M.; Tan, E. M. Histopathological changes in mice treated with extracts of the histamine-sensitizing factor of *Bordetella pertussis*. Int. Arch. Allergy Appl. Immunol. 54:129–142; 1977.

15. Goudie, R. B.; Home, C. H. W.; Wilkinson, P. C. A simple method for producing antibody specific to a single selected diffusible antigen. Lancet ii; 1224; 1966.

16. Grabar, P.; Williams, C. A. Methode permettant l'etude conjugee des proprietes electrophoretiques et immunochimiques d'un melange de proteines. Application au serum sanguin. Biochim. Biophys. Acta 10:193; 1953.

17. Reisfeld, R. A.; Small, P. A. Electrophoretic heterogeneity of polypeptide chains of specific antibodies. Science 152:1253–1255; 1966.

Islet-Activating Protein in *Bordetella pertussis*: Purification and Mechanism of Action

M. Ui, T. Katada, and M. Yajima

ABSTRACT

A new protein purified from the culture medium of *Bordetella pertussis* was named islet-activating protein (IAP). IAP has a molecular weight of 77,000 and consists of three different subunits, which were separated by incubation with 8M urea. None of the subunits had biological activity. Incubation of the smallest subunit with either of the other two resulted in a new association product as active as native IAP. IAP at a dose as low as 1 μg was effective in potentiating insulin secretory responses of rat pancreas over a period of 3 to 60 days after a single administration. On the basis of experimental results with pancreatic islets isolated from IAP-treated rats or with normal rat islets cultured for 24 hours with IAP at concentrations above 0.1 ng/ml, it was concluded that the IAP molecule interacts directly but slowly with the islet B-cell to cause sustained activation of native calcium ionophores on the cell membrane, which in turn makes more calcium available to the stimulus-secretion coupling mechanism.

INTRODUCTION

It has been shown in our laboratory that rats given a single intraperitoneal injection of pertussis vaccine (10^{11} organisms per 100 g body weight) exhibited markedly enhanced hyperinsulinemia in response to insulin secretagogues such as glucose, sulfonylureas, and β-adrenergic agents (1,2). Epinephrine, an α- and β-adrenergic agent, which inhibited glucose-induced hyperinsulinemia in normal rats via α-adrenergic receptors, enhanced the hyperinsulinemia in pertussis-sensitized rats by stimulating β-adrenergic receptors. Epinephrine-induced hyperglycemia frequently has been reported to be attenuated in pertussis-sensitized rats. It was accounted for solely by the hypoglycemic action of insulin secreted in sensitized rats in response to β-adrenergic action of the catecholamine (3).

Neither the number and microscopic structure of pancreatic islets nor their insulin content was affected by pertussis sensitization. Nevertheless, more insulin was released from pancreas of pertussis-sensitized rats than from pancreas of normal rats during perfusion with various insulin secretagogues (4). Representative insulin secretion profiles are illustrated in the panels of Figure 1. Roughly twice as much insulin was released from pancreas of pertussis-sensitized rats than from pancreas of normal rats when the organ was perfused with a high concentration (13.3 mM) of glucose (Panels A, B, and C in Figure 1). In the case of pancreas

from normal rats, the addition of epinephrine to perfusate caused a strong inhibition of the glucose-induced secretion (Panel A) due to stimulation of α-adrenergic receptors, as evidenced by a reversal of the inhibition on simultaneous addition of an α-adrenergic antagonist (Panel C). In sharp contrast, epinephrine alone caused a marked secretion of insulin from the pancreas of pertussis-sensitized rats (Panel A). An involvement of β-adrenergic receptors in the secretion was clearly indicated by the failure of the catecholamine to cause insulin secretion in the presence of a β-adrenergic antagonist (Panel B).

Thus prior treatment of rats with pertussis vaccine exerted a profound and sustained influence on an insulin secretory machinery in the pancreatic B-cell. A new protein responsible for this unique action of the vaccine was recently purified from the culture medium of *Bordetella pertussis* and was named "islet-activating protein (IAP)" (5,6). In this paper we summarize the purification, characterization, and properties of IAP and describe the mechanism by which it potentiates insulin secretory responses of rats.

MATERIALS AND METHODS

Purification of IAP

Bioassay of IAP activity. For assay of IAP activity the test solutions (suspensions of microorganisms, supernatants of culture media, or protein fractions
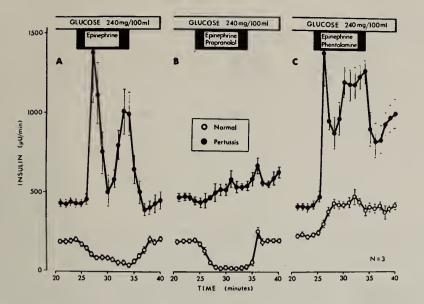
Figure 1. Effect of epinephrine with or without adrenergic antagonists on insulin release from perfused pancreas of normal (Normal) or pertussis-sensitized (Pertussis) rats. Pancreas was perfused with Krebs-Ringer bicarbonate solution supplemented with 1% bovine serum albumin and 13.3 mM glucose. Duration of infusion of epinephrine (0.6 μM) alone or with propranolol (a β-adrenergic antagonist, 38 μM) or phentolamine (an α-adrenergic antagonist, 30 μM) is shown by a solid block.

obtained during purification) were injected intravenously into rats. Three days later, the rats were given an intraperitoneal glucose load (300 mg/100 g body weight) after 20 hours of fasting. Plasma concentrations of glucose and insulin were determined immediately before and 15 minutes after the glucose load. The ratio of an increment of insulin (ΔIRI) to an increment of glucose (ΔGlc) was calculated for each rat and was averaged within a group of three rats pretreated with the same amount of test solution. The percent increase of this value over the value averaged for a group of three control rats pretreated with saline was used to define units (IAP units) of IAP activity. According to this definition, 100 IAP units represent the activity that doubles the insulin secretory response of rats to a given degree of hyperglycemia. The IAP unit thus defined was found to be proportional over a wide range of IAP activity (5).

Source of IAP. Cells of *B. pertussis,* phase I, exhibited IAP activity; no activity was detected in the phase III cells and other species of *Bordetella* tested (5). The most potent activity was present in the supernatant of a 48-hour culture, which was therefore used as the starting material for subsequent purification.

Purification procedure. IAP was purified by sequential chromatography on columns of hydroxyapatite, carboxymethylagarose (CM-Sepharose CL–6B), and concanavalin A–Sepharose (5). After being loaded with crude protein solution, each column was washed with phosphate buffer (0.01 M, pH 6.0). The protein fractions with IAP activity were eluted as a sharp peak with a phosphate buffered

(0.1 M, pH 7.0) solution of 0.5 M NaCl. The eluate was concentrated in vacuo and dialyzed against phosphate buffer (0.01 M, pH 6.0) before being applied to the next column. Final purification was achieved by gel filtration through a column of Biogel P–100 with a mixture of phosphate buffer (0.01 M, pH 6.0) and 0.5 M NaCl. The purification procedure is summarized in Table 1. The yield was high; roughly 15–20% of the total IAP activity contained in the supernatant of culture media was recovered as a homogeneous protein (see below) at a specific activity 1,350 times that of the original protein.

RESULTS

Chemical and Physical Properties of IAP

IAP thus purified produced a single band of protein in polyacrylamide gel electrophoresis (Fig. 2) and a single precipitation arc with anti-IAP antiserum in Ouchterlony double immunodiffusion (Fig. 3). Its molecular weight was estimated as 77,000 by gel filtration through a column of Sephadex G–100. The carbohydrate content was 1.0–1.5%, though there was no evidence for the presence of amino sugars. Lipid was not detected before or after alkaline hydrolysis. Representative amino acid analysis data will be shown later (see Table 3).

IAP was soluble in water, and solutions were stable at temperatures below 37° C unless exposed to a pH lower than 4 or higher than 9.

Biological Activity of IAP

Units of activity determined as above are plotted against the logarithm of the amount of purified

Table 1. Summary of Purification Procedure for IAP

Steps	Total Protein (mg)	Total Activity (units × 10⁻⁶)	Specific Activity (units/µg)	Yield (%)	Purification
Supernatant of culture medium (10 l)	22,000	14	0.64	100	1
Hydroxyapatite column (2.6 × 4 cm) a	15.2	5.9	386	42	610
CM-Sepharose column (1.5 × 10 cm)	7.5	4.8	642	34	1,010
Con-A-Sepharose column (1.5 × 8 cm)	5.0	3.8	760	27	1,200
Biogel P–100 column (2.8 × 60 cm)	2.8	2.4	858	17	1,350

a The column size is shown in parentheses. Elution from columns was carried out at a flow rate of 20 ml/h, except for the column of Biogel P–100, which was eluted at a flow rate of 30 ml/h.

Figure 2. Polyacrylamide gel electrophoresis of the purified IAP.
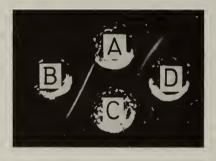
Figure 3. Immunodiffusion pattern in agar gel. A, IAP; B, anti-pertussis antiserum (fourfold dilution); C, condensed culture fluids (30 mg protein/ml); D, anti-IAP antiserum.

IAP (from 20 ng to 2 µg) in Figure 4. IAP injected into rats at a dose as low as 1–2 µg per animal exhibited maximal activity and caused a 10- to 12-fold potentiation of the insulin secretory response to glucose (5). The response of rats to other insulin secretagogues in vivo was also potentiated by pretreatment of the animal with IAP.

The duration of IAP activity is shown in Figure 5. After IAP at the doses indicated was injected intravenously into rats, their insulin secretory response to glucose loading was measured at the times shown on the abscissa (6). When rats were injected with 1–2 µg of IAP, maximum potentiation of the insulin secretory response was observed over a period of 3 to 10 days. The degree of potentiation decreased rapidly afterwards, but there were still fivefold and threefold increases in insulin secretion over the control value 3 and 6 weeks respectively after the injection. Even 2 months later, twice as much insulin was secreted in the IAP-
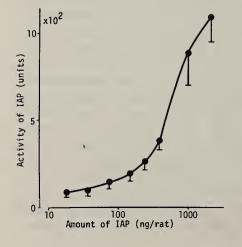

Figure 4. Dose-response curve of purified IAP injected into rats. Purified IAP was injected intravenously into rats at the doses indicated on the abscissa. Responses of these rats to intraperitoneal glucose load, 3 days after the IAP injection, were used for calculation of IAP activity units as described in text.
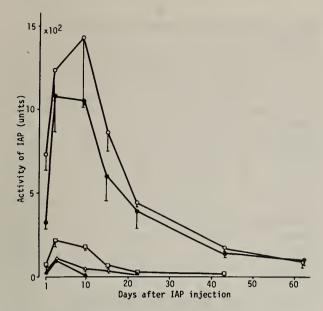
Figure 5. Duration of the IAP activity units on insulin secretory responses of rats. The IAP activity units were determined at various intervals after injection of IAP (IAP injection: *day-0*) as an index of insulin secretory response to a glucose load. The dose of IAP was: ▲, 8 ng; △, 32 ng; □, 125 ng; ●, 1 μg; ○, 2 μg. Each point with a vertical line shows the mean± SEM from three animals.
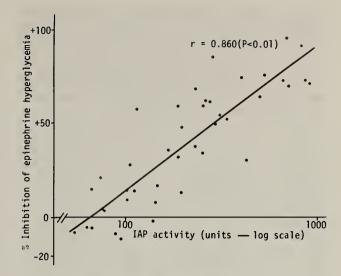
Figure 6. Correlation between the logarithm of IAP activity units and percent inhibition of epinephrine-induced hyperglycemia. IAP was injected intravenously into rats on the first day of the experiment. On the second day, the rats were injected subcutaneously with epinephrine (20 μg/100 g body wt) to obtain the percent inhibition of epinephrine hyperglycemia. On the fourth day, the rats were again injected intraperitoneally with glucose (0.2 g/100 g body wt) to determine the IAP activity units. The rats were fasted for 20 hours before challenge with epinephrine or glucose. These two parameters for each rat are shown on the ordinate and abscissa, respectively, for 44 rats treated with various doses of IAP.

treated rats as in the control rats in response to a glucose load. As the dose of IAP was reduced, the duration of its action was also reduced.

The pretreatment of rats with IAP led to an inhibition of epinephrine-induced hyperglycemia. The rats that were used for estimation of IAP units have been studied for their response to epinephrine hyperglycemia, and the degree of hyperglycemia was compared with that observed in the control rats that had been treated with saline instead of IAP. As shown in **Figure 6**, a highly significant ($p<0.01$) correlation was found to exist between the percent inhibition of epinephrine hyperglycemia and the IAP activity (determined on the same rat) over a wide range of IAP doses (6). Moreover, epinephrine caused the same degree of hyperglycemia in IAP-treated rats as in control rats when endogenous insulin was neutralized with anti-insulin serum or when the pancreas had been depleted of insulin by induction of streptozotocin diabetes (6). Thus hyperinsulinemia from IAP activity is solely responsible for the attenuation of epinephrine-induced hyperglycemia in IAP-treated rats.

Like the original pertussis vaccine, IAP caused leukocytosis and increased sensitivity to lethal doses of histamine in mice. The relative potencies of these biological activities were estimated in mice in

which the ability to inhibit epinephrine-induced hyperglycemia was used as an index of IAP activity on the basis of the above conclusion. IAP at doses higher than 0.5 μg per animal induced severe leukocytosis, increased the number of deaths due to histamine, and inhibited epinephrine hyperglycemia (Table 2). When the dose of IAP was lower than 0.1 μg, however, there was no leukocytosis and no increased mortality from histamine, despite significant attenuation of epinephrine-induced hyperglycemia (6). It is very likely, therefore, that lower doses of IAP only potentiate insulin secretory responses and that leukocytosis is not responsible for this action of IAP. In the case of histamine challenge, a high dose such as that used in this experiment caused marked hyperinsulinemia in IAP-treated mice, even though it was ineffective in non-treated mice. Hyperinsulinemia leads to hypoglycemia, which conceivably exaggerates the toxic action of histamine. Thus IAP also appears to be responsible for enhanced histamine sensitivity, which has been frequently reported to occur in mice treated with pertussis vaccine or with proteins isolated from it.

Table 2. Number of White Blood Cells, Histamine-Induced Deaths and Glycemic Response to Epinephrine of Mice Treated with IAP. Experiments were carried out with fasted mice which had been injected intravenously with IAP 3 days before. Blood glucose was determined immediately before and 30 minutes after an intraperitoneal injection of epinephrine (5 μg/mouse). Means $\pm$ SEM from five animals are recorded for leukocyte numbers and blood glucose values.

Dose of IAP (μg)	White Blood Cells (cells $\times$ 10⁵/ml)	Histamine Death (/total)	Increase in Blood Glucose Induced by Epinephrine [a] (mg/100 ml)
0 (control)	129±8.0	0/5	72±1.3
0.02	147±5.4	0/5	29±9.2 [b]
0.1	138±5.4	0/5	22±3.1 [b]
0.5	501±24.6 [b]	1/5	1±10.4 [b]
1.0	721±34.0 [b]	3/5	−20±2.7 [b]

[a] A minus sign represents a decrease.

[b] Difference from control is statistically significant (p<0.01).

Subunit Structure of IAP

The biological activity of IAP was completely lost during a 2-hour incubation with 8 M urea at 37° C (7). After incubation, the protein solution was applied to a column of Sephacryl S–200, from which three fractions of protein were eluted in the presence of 8 M urea. These subunits of IAP were purified individually by means of ion exchange chromatography and were designated F–1, F–2, and F–3 in decreasing order of molecular weight. Migration profiles of these subunits on sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of 1% 2-mercaptoethanol are shown in Figure 7. The F–1 subunit consisted of two polypeptide chains with molecular weights of 24,000 and 20,000, while the F–2 and F–3 subunits were single-chain proteins with molecular weights of 20,000 and 11,000 respectively. The amino acid compositions of these subunits, together with those of the native IAP, are shown in Table 3. The

Table 3. Amino Acid Compositions of IAP and Its Subunits

Amino Acid	Residues (% of total)			
	F–1	F–2	F–3	IAP
Aspartic acid	8.1	9.3	5.3	7.5
Threonine	8.5	7.7	4.8	7.3
Serine	7.9	9.8	6.2	6.4
Glutamic acid	9.6	11.8	9.6	10.0
Proline	5.0	4.6	9.3	5.8
Glycine	10.0	9.8	8.1	8.8
Alanine	9.5	11.3	9.3	9.3
1/2 Cystine	1.1	n.d.[a]	2.3	2.5
Valine	6.2	7.4	10.2	6.5
Methionine	1.8	1.9	6.0	2.8
Isoleucine	4.5	4.0	2.2	4.0
Leucine	6.5	5.3	8.6	7.8
Tyrosine	7.6	6.2	2.5	6.5
Phenylalanine	2.8	2.5	4.5	3.7
Lysine	2.1	1.9	5.8	3.3
Histidine	1.6	1.1	0.5	1.5
Arginine	7.3	5.4	4.8	6.4

[a] Not detected

molar ratio of F–1, F2, and F–3 in native IAP is 1:1:2.

Although none of these subunits was biologically active by itself, the equimolar combination of F–3 with either F–1 or F–2 produced essentially the same biological activity as native IAP on a molar basis (Table 4). In contrast, the combination of F–1 with F–2 was inactive. Each of these combinations with the F–3 subunit, after 24 hours incuba-

Table 4. Biological Activities of IAP Subunits and Their Combinations. Subunits, individually or in combination as shown, were incubated in 0.1 M phosphate buffer (pH 7.0) at 37° C for 24 hours. After incubation, the reaction mixture was diluted so that the total amount of protein was 2 μg per animal. Biological activities were determined as in Table 2.

F–1	F–2	F–3	IAP Activity (units $\times$ 10⁻²)	Inhibition of Epinephrine Hyperglycemia (% inhibition)	Leukocytosis Induction (times control)	Histamine Death (death/total)
−	−	−	− [a]	−	−	0/3
+	−	−	−	−	−	0/3
−	+	−	−	−	−	0/3
−	−	+	−	−	−	0/3
+	+	−	−	−	−	0/3
+	−	+	9	90	4.5	3/3
−	+	+	11	87	3.9	3/3
+	+	+	12	107	4.2	3/3

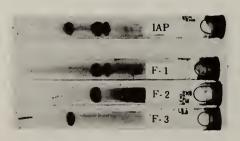[a] The biological activity observed was not significant.

Figure 7. Migration profiles of IAP and its subunits on sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of 1% 2-mercaptoethanol.

tion at 37° C, showed a single peak on gel filtration or polyacrylamide gel electrophoresis (Fig. 8), indicating the generation of new association products (8). Thus the active center for the biological activity of IAP appears to be on the F-3 subunit, though its association with either of the inactive subunits, F-1 or F-2, is required for biological activity. Further combination of these two kinds of active association products at the molar ratio of 1:1 is likely to form the native IAP molecule (8).

Mechanism of IAP Action [1]

Islets isolated by a collagenase method from the pancreas of IAP-treated or untreated rats were incubated in the presence of various secretagogues (Table 5). Enhancement of insulin release induced

Figure 8. Polyacrylamide gel electrophoresis of native IAP, S-13 (an association product of F-3 with F-1) and S-23 (an association product of F-3 with F-2).

[1] Katada, T.; Ui, M. lslet-activating protein: enhanced insulin secretion and cyclic AMP accumulation in pancreatic islets due to activation of native calcium ionophores. J. Biol. Chem. In press, 1978. Ui, M.; Katada, T. A novel action of islet-activating protein (IAP) to modify adrenergic regulation of insulin secretion. Baba, S.; Kaneko, T.; Rubenstein, A. H.; Steiner, D. F.; Yanaihara, N., eds. Symposium on proinsulin, insulin, and C-peptide. Amsterdam: Excerpta Medica; 1978. In press.

by insulin secretagogues such as glucose, glucagon, arginine, and 3-isobutyl-1-methylxanthine (IBMX) was associated with more accumulation of cyclic AMP in islet cells, indicating that insulin release was mediated by cyclic AMP generation.

Pretreatment of pancreas donor rats with IAP potentiated the insulin release induced by these secretagogues, although it did not affect the basal rate of insulin release observed without any secretagogues. The potentiation of insulin release by IAP treatment was not associated with increased accumulation of cyclic AMP, unless the breakdown of cyclic AMP was inhibited by the potent phosphodiesterase inhibitor, IBMX. Thus we conclude that IAP treatment not only activates the generation of cyclic AMP in islet cells of rats but also activates its breakdown by phosphodiesterase. An additional site of IAP action is conceivably located distal to cyclic AMP generation in the overall process of stimulus-secretion coupling in the B-cell, because more insulin was released after IAP treatment despite unchanged cyclic AMP content in islet cells not supplemented with IBMX.

Both insulin release and cyclic AMP accumulation induced by the addition of 16.7 mM glucose were strongly inhibited by epinephrine in a dose-dependent manner in islets of normal rats (Fig. 9). No inhibition was observed, however, when an α-adrenergic antagonist was added simultaneously, which is evidence that the epinephrine-induced inhibition was mediated by stimulation of α-adrenergic receptors. In accordance with the results of perfusion experiments (Fig. 1), this α-receptor-mediated

Table 5. Insulin Release and Cyclic AMP Contents in Pancreatic Islets After Incubation with Various Isulin Secretagogues. Islets from IAP-treated (IAP) or untreated (normal) rats were incubated in the basal medium for 30 min at 37° C. IAP-treated rats were prepared by injecting 1 μg of IAP intravenously 3 days before sacrifice.

Additions	No. of Observations	Insulin Release		Cyclic AMP	
		Normal	IAP	Normal	IAP
		(μU/islet)		(fmol/islet)	
Without IBMX					
None [a]	8	9±0.9	8±0.4	13±0.7	13±0.7
Glucose (10 mM)	4	24±1.2	68±4.9 [b]	25±2.3	26±2.4
Arginine (0.13 mM)	4	21±2.5	36±3.2 [b]	16±2.2	17±1.6
Glucose +glucagon (0.35 μM)	4	74±3.5	131±6 [b]	43±4.0	36±2.0
With IBMX (1 mM)					
None [a]	6	26±1.5	99±3.9 [b]	73±3.5	199±8 [b]
Glucose	4	195±15	290±17 [b]	146±18	336±54 [b]
Glucose +glucagon	4	247±14	368±24 [b]	240±21	657±43 [b]

[a] Basal incubation medium: Krebs-Ringer bicarbonate solution supplemented with 0.5% albumin, 400 KIU/ml of aprotinin and 3.3 mM glucose.
[b] Effect of IAP is significant (p<0.01).

inhibition was no longer observable in islets isolated from IAP-treated rats (Fig. 9).

The presence of calcium in the incubation medium was indispensable for islets of IAP-treated rats to exhibit the unique properties of releasing insulin and accumulating cyclic AMP at higher-than-normal rates without being suppressed via α-adrenergic receptors. When calcium was omitted from the incubation medium, there were essentially no differences in insulin release and cyclic AMP accumulation between islets of normal rats and of IAP-treated rats even in the presence of IBMX (Fig. 10). Furthermore, epinephrine was inhibitory in islets from IAP-treated rats unless the incubation medium was fortified with calcium. The translocation of calcium through the cell membrane was next studied by incubating ^{45}Ca-loaded islets in the medium supplemented with nonradioactive calcium. Translocation of calcium was accelerated by pretreatment of the islet donor rats with IAP (Fig. 11). Thus the action of IAP appears to be associated with enhanced influx of extracellular calcium into the pancreatic B-cell.

Figure 10. Significance of extracellular calcium for the difference in insulin secretory responses between islets of normal and IAP-treated rats. Islets were incubated with 16.7 mM glucose in the presence or absence of IBMX as in Figure 9. The presence and absence of epinephrine (0.7 μM) and CaCl$_2$ (2.5 mM) are indicated by + and − respectively at the bottom.
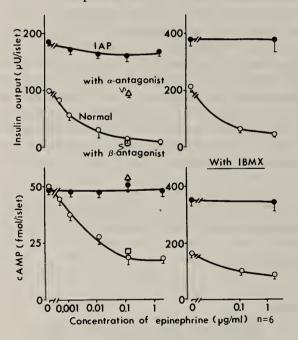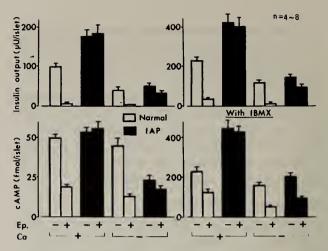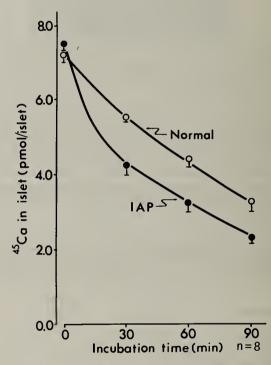

Figure 9. Insulin release and cyclic AMP accumulation in islets from normal or IAP-treated rats. Islets from fasted normal (open symbols) or IAP-treated (solid symbols) rats were incubated with 16.7 mM glucose for 30 min in the presence (left-hand panels) or absence (right-hand panels) of 0.5 mM IBMX. Epinephrine was added as indicated alone (circles) or with either 8.7 μM phentolamine (open triangles) or 6.6 μM propranolol (open squares). The islet content of cyclic AMP after incubation was determined by the sensitive method of Honma et al. (9) in this and other figures and tables.

Figure 11. Efflux of ^{45}Ca from islets of normal and IAP-treated rats. Islets were loaded with ^{45}Ca, and the ^{45}Ca efflux in exchange with nonradioactive Ca was estimated during incubation for 90 min. Ca-nondisplaceable ^{45}Ca retained in islets is plotted as a function of incubation time in the nonradioactive medium.

In the foregoing experiments, pancreases were isolated from rats that had been injected with IAP 3 days before. It took at least 1 day before the effects of IAP could be observed in the whole ani-

mal or with pancreatic preparations. In agreement with these effects in vivo, IAP was without effect when added in vitro into perfusates or incubation media of pancreatic preparations. In order to study if IAP directly exerts its influences on islet cells in vitro as progressively as in vivo, isolated islets were cultured for 24 hours in the presence or absence of IAP. The cultured islets were then washed and further incubated for 30 minutes in the fresh medium supplemented with 16.7 mM glucose. As Figure 12 shows, more insulin was released in response to 16.7 mM glucose from islets cultured with IAP at concentrations above 1 ng/ml than from islets cultured without IAP. Epinephrine became less inhibitory to insulin release as the concentration of IAP during cell culture was increased from 0.1 ng/ml to 100 ng/ml. No inhibition was observed with 10 or 100 ng/ml of IAP. Thus IAP is capable of directly influencing pancreatic islet cells.

DISCUSSION

On the basis of these results, the mechanism by which IAP potentiates insulin secretory responses of pancreas can be summarized as follows. The molecule of IAP, more exactly its F-3 subunit associated with the F-1 or F-2 subunit, directly interacts with the cell membrane of the islet B-cell to cause a sustained and progressiv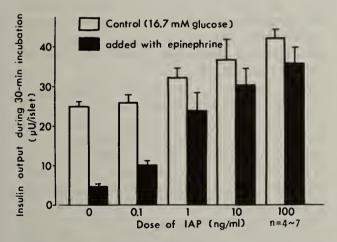e activation of native calcium ionophores located in the membrane. As a result, more calcium becomes available in an intracellular pool, which not only acts as a trigger of insulin secretion but also activates adenylate cyclase and phosphodiesterase.

Native calcium ionophores are conceivably close to, or coupled functionally with, α-adrenergic receptors on the cell membrane, since activation of the ionophores leads to blockage of the receptors whereas stimulation of the receptors inhibits translocation of calcium across the ionophores. Thus activation of the native ionophores by IAP treatment reversed α-adrenergic inhibition of insulin release and cyclic AMP generation. Since the α-adrenergic inhibition was caused in vitro by a low concentration of epinephrine comparable to its plasma concentration in vivo, it is highly probable that the insulin secretory responses of pancreas in vivo are suppressed by the α-adrenergic action of endogenous epinephrine, and that a reversal of the suppression would also result in potentiation of insulin secretory responses in general.

REFERENCES

1. Sumi, T.; Ui, M. Potentiation of the adrenergic *beta*-receptor-mediated insulin secretion in pertussis-sensitized rats. Endocrinology 97:352–358; 1975.
2. Katada, T.; Ui, M. Spontaneous recovery from streptozotocin-induced diabetes in rats pretreated with pertussis vaccine or hydrocortisone. Diabetologia 13:521–525; 1977.
3. Katada, T.; Ui, M. Accelerated turnover of blood glucose in pertussis-sensitized rats due to combined actions of endogenous insulin and adrenergic *beta*-stimulation. Biochim. Biophys. Acta 421:57–69; 1976.
4. Katada, T.; Ui, M. Perfusion of the pancreas isolated from pertussis-sensitized rats: potentiation of insulin secretory responses due to β-adrenergic stimulation. Endocrinology 101:1247–1255; 1977.
5. Yajima, M.; Hosoda, K.; Kanbayashi, Y.; Nakamura, T.; Nogimori, K.; Nakase, Y.; Ui, M. Islet-activating protein (IAP) in *Bordetella pertussis* that potentiates insulin secretory responses of rats: purification and characterization. J. Biochem. (Tokyo): 83:295–303; 1978.
6. Yajima, M.; Hosoda, K.; Kanbayashi, Y.; Nakamura, T.; Takahashi, I.; Ui, M. Biological properties of islet-activating protein (IAP) purified from the culture medium of *Bordetella pertussis*. J. Biochem. (Tokyo) 83:305–312; 1978.
7. Kanbayashi, Y.; Nakamura, T.; Hosoda, K.; Nogimori, K.; Yajima, M.; Ui, M. Subunit structure of islet-activating protein (IAP), a new protein isolated from the culture media of *Bordetella pertussis*. J. Biochem. (Tokyo) 84:443–451; 1978.
8. Kanbayashi, Y.; Nakamura, T.; Hosoda, K.; Nogimori; K.; Yajima, M.; Ui, M. Formation of biologically active protein from the subunits of islet-activating protein(IAP), a new protein isolated from *Bordetella pertussis*. J. Biochem. (Tokyo) 84:453–460; 1978.
9. Honma, M.; Satoh, T.; Takezawa, J.; Ui, M. An ultrasensitive method for the simultaneous determination of cyclic AMP and cyclic GMP in small-volume samples from blood and tissue. Biochem. Med. 18:257–273; 1977.

Figure 12. In vitro effect of IAP on cultured islets. Islets prepared from normal fasted rats were cultured in the TCM–199 medium fortified with 10% fetal calf serum, 16.7 mM glucose, and antibiotics at 37°C for 24 hours under an atmosphere containing 5% CO_2. IAP was added during the culture at concentrations indicated. Islets were then washed several times and further incubated in a fresh Krebs-Ringer bicarbonate solution containing 16.7 mM glucose for 30 minutes in the presence (solid bars) or absence (open bars) of 0.7 μM epinephrine.

Bordetella pertussis Extracellular Adenylate Cyclase and its Biological Activity

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ABSTRACT

Our investigation has shown that extracellular adenylate cyclase (EC.4.6.1.1) occurs in supernatants of *B. pertussis* fluid cultures and in charcoal agar and Bordet-Gengou medium as well. After cultures were harvested, the charcoal agar and Bordet-Gengou medium were frozen overnight, thawed, and centrifuged. The supernatant was precipitated by ammonium sulfate. Extracellular adenylate cyclase, concentrated in the precipitate, was dissolved in phosphate buffer, pH 8.0 containing M NaCl.

During the exponential growth phase, *B. pertussis* grown in liquid medium released adenylate cyclase into the supernatant fluid. This release was greater in cultures which contained amberlite resin than in those without it. A comparison was made of the amount of adenylate cyclase contained in supernatant fluids from pertussis vaccines prepared from cultures grown in fluid or on solid media.

Several toxicity tests were run with crude preparations to determine the relationship of adenylate cyclase to other biological activities of *B. pertussis*. The preliminary results suggested that adenylate cyclase has no toxicity. Adenylate cyclase (0.8 mg protein/ml) had no effect in the paw edema test, mouse weight gain test, and rabbit intradermal test (no necrosis). In the rabbit dermonecrosis test, however, signs of delayed hypersensivity appeared (hyperemia 2–3 days post-injection).

It was demonstrated that no serological relationship exists between agglutinogens 2 and 3 and adenylate cyclase.

INTRODUCTION

Adenosine-3′,5′-cyclic monophosphate (aAMP), the product of the enzyme adenylate cyclase (EC 4.6.1.1), participates in a series of reactions essential for cell metabolism: catabolic suppression, reactions of phosphorylation, and dephosphorylation. In eukaryotes adenylate cyclase and cAMP are mediators, which together with different hormones influence cell metabolism. Some bacterial products which stimulate or inhibit adenylate cyclase, thus affecting eukaryotic cells, have been isolated.

In recent studies in 21 species of microorganism (*B. liquefaciens, E. coli, M. lysodeikticus, A. globoformis,* and others) adenylate cyclase was associated with the cytoplasmic membrane. Hewlett et al. (1) showed that during cultivation of *B. pertussis* there was a release of free adenylate cyclase into the fluid medium. This paper presents various conditions which lead to the formation of *B. pertussis* extracellular adenylate cyclase and its properties.

METHODS

Bordetella pertussis strain 41405 (Phase I), *B. parapertussis* strain 17903, and *B. bronchiseptica* strain 8344 were cultivated on Bordet-Gengou medium as well as on nonsynthetic solid (Z_4) and synthetic fluid (Z_{15}) media.

Solid medium Z_4 consisted of tryptose (Difco), 20 g; NaCl, 5 g; soluble starch (Difco), 10 g; L-proline (Merck), 0.45 g; L-glutamic acid (Merck), 0.25 g; nicotinamide (Koch-Light), 50 mg; L-cystine hydrochloride (Merck), 50 mg; L-ascorbic acid (Merck), 20 mg, Noble agar (Difco), 20 g; distilled water, 1 liter, pH 7.4.

Fluid medium Z_{15} consisted of sodium L-glutamate (Merck), 10.72 g; NaCl, 2.5 g; $KH_2 PO_4$, 0.5 g; KCl, 0.2 g; $MgCl_2$, 0.1 g; $CaCl_2$, 0.02 g; DL-proline (Koch-Light), 0.48 g; Tris (hydroxymethyl)-aminomethane (Fluka), 1.525 g; L-cysteine (Merck), 40 mg; $FeSO_4$, 10 mg; nicotinic acid (Merck), 4 mg; glutathione-reduced form (Merck), 100 mg; distilled water, 1 liter, pH 7.4.

After 48 hours incubation at 36° C, bacterial cultures were harvested in a 2% solution of casamino acids (Difco). This suspension (OD_{650} = 0.080) was seeded in 0.5 ml aliquots on solid medium in large petri dishes (20 cm) or in 2 ml aliquots into 2 liter Roux flasks, each containing 100 ml of fluid medium Z_{15}. The flasks were incubated in the horizontal position at 36° C. After 24 or 48 hours of

incubation the cultures were centrifuged 20 minutes at 5,000 × g at 4° C, the supernatant fluid was filtered through a Millipore filter (0.22 μm) and the adenylate cyclase activity was determined.

For solid medium cultures, bacteria were cultivated for 48 hours at 36° C on Z_4 or Bordet-Gengou medium. Cultures were harvested by covering the surface with saline and rubbing the surface of the medium with cotton wool. To determine adenylate cyclase activity, the medium was then cut into pieces 1—2 cm in size and frozen. After 16 hours, the pieces were thawed at 30° C for 60 minutes and centrifuged for 30 minutes at 20,000 × g (4° C). The supernatant fluid was precipitated with ammonium sulfate (33% saturation) and left overnight at 4° C. The precipitate was pelleted by centrifugation for 15 minutes at 10,000 × g (4° C) and resuspended in 0.05 M phosphate buffer, pH 8.0, with M NaCl and again centrifuged for 20 minutes at 14,000 × g (4° C). The supernatant was dialyzed against 0.06 M Tris buffer, pH 7.4, for 2 days, then assayed for adenylate cyclase activity.

The enzyme activity was estimated by the increase in the amount of cAMP after incubation for 30 minutes at 35° C utilizing the modified method of Brown (2): 0.02 ml of the test preparation was added to 0.04 ml of a mixture containing 0.01 M theophylline (Merck), 0.01 M $MgCl_2$, 0.1% bovine albumin (Sigma) and 0.002 M ATP (Sigma). After incubation 0.05 ml of ³H-cAMP (Amersham) (40 pM, about 10,000 cpm) and 0.1 ml of cAMP-binding protein were added to the mixture. cAMP-binding protein was obtained from rabbit muscles by the method of Walsh (3). After incubation for 100 minutes at 4° C, 0.1 ml of a mixture containing 10% activated charcoal (Norit, Merck) and 2% bovine albumin (Sigma) in 0.05 M Tris buffer with 0.004 M EDTA (pH 7.5) was added. The reaction mixture was centrifuged for 2 minutes at 5,000 × g (4° C) and 0.2 ml of the supernatant fluid was mixed with 0.8 ml distilled water and 5 ml scintillation cocktail (butyl-PBD, POPOP, and dioxan). Radioactivity was measured by a liquid scintillation spectrometer SL-40 (Intertechnique—France). The adenylate cyclase activity was estimated by the method of Gilman (4) and expressed as picomoles cAMP/mg protein/hour.

Results and Discussion

In fluid medium Z_{15}, maximum production of extracellular adenylate cyclase occurred during the exponential growth phase. Addition of one percent (w/v) of the anion exchange resin amberlite IR–45 or IRA–410 to the medium raised the cyclase activity (Table 1). This effect was detectable after 24 hours, but was more pronounced after 48 hours of cultivation, especially in the presence of weakly alkaline anionic amberlite IR–45.

During cultivation in Z_{15} medium with amberlite, the cyclase activities of B. parapertussis and B. bronchiseptica were respectively 1/40 and 1/10 that of B. pertussis (Table 2).

High adenylate cyclase activity was also elicited in the dialyzed extracts from solid Z_4 and Bordet-Gengou media. Apparently Bordetella species secrete the enzyme when grown on fluid and solid media of different composition. The extracellular adenylate cyclase from Bordetella species is a relatively stable enzyme. It is inactivated at 50° C and the energy of inactivation is 11,000 cal. This property distinguishes it from analogous intracellular cyclases isolated from other species of microorganisms and eukaryotic cells.

Along with these investigations, we determined adenylate cyclase levels in vaccines prepared by two laboratories from ten production strains of B. pertussis grown on casein charcoal agar (in Moscow) and fluid medium with Wofatut's resin (in Sofia). All vaccines were inactivated with 0.1% formalin. Similar determinations were done with three lots of DTP adsorbed final product vaccine.

The adenylate cyclase content was very low (from 0.01 to 1.17 picomoles cAMP/min/immunizing dose) in pertussis vaccines prepared on solid medium. No adenylate cyclase was found in the three

Table 1

Media	OD$_{650}$		Protein		Enzyme Activity [a]	
	24 h	48 h	24 h	48 h	24 h	48 h
IR-45	0.205	0.708	1.57	1.59	1,044	2,025
IRA-410	0.268	0.591	1.59	1.66	1,284	426
Z_{15}	0.224	0.303	0.44	0.45	705	60

[a] In picomoles cAMP/ml/hour

Table 2

Strain	Enzyme Activity (picomoles cAMP/ml/min)
B. pertussis 41405	320
B. parapertussis 17903	8
B. bronchiseptica 8344	34

lots of DTP adsorbed vaccine prepared from the same suspensions. In pertussis suspensions prepared from fluid culture, the adenylate cyclase content ranged from 6.09 to 64.56 pM cAMP/min/immunizing dose (Table 3). The results support the above data on the ability of anionic resins to enhance the release of extracellular adenylate cyclase.

Several toxicity tests were performed with crude preparations to determine the significance of extracellular adenylate cyclase on biological activities of *B. pertussis*. Preliminary results suggest that extracellular adenylate cyclase has no toxic effects. Adenylate cyclase from *B. pertussis* (0.8 mg protein/ml)

has no effect in the paw edema test, mouse weight-gain test, and rabbit intradermal test (no necrosis). In the rabbit dermonecrosis test, however, signs of delayed hypersensitivity appeared (hyperemia 2–3 days post-injection) and lasted several days.

Serological studies showed that enzyme preparations do not react with specific antibacterial pertussis serum by precipitation and do not absorb agglutinins to agglutinogens 2 and 3 from monospecific sera. Extracellular adenylate cyclase is not related to agglutinogens 2 or 3.

Additional studies are in progress to evaluate the importance of the data presented here.

Table 3

Solid Medium		Fluid Medium	
B. pertussis strain	Enzyme Activity [a]	*B. pertussis* strain	Enzyme Activity [a]
475	0.37	475	6.09
305	0.83	305	10.08
703	1.17	358	64.56
GL 353	0.33	41,405	33.00
267	0.17		
134	0.008		
38	0.37		
509	0.58		

[a] In picomoles cAMP/ml suspension 2×10^{10} cells/min

REFERENCES

1. Hewlett, E.; Urban, M.; Manclark, C.; Wolff, J. Extracytoplasmic adenylate cyclase of *Bordetella pertussis*. Proc. Natl. Acad. Sci. USA 73:1926–1931; 1976.
2. Brown, B.; Albano, J.; Ekins, R.; Sgherzi, A. A simple and sensitive saturation method for the measurement of adenosine 3′,5′-cyclic monophosphate. Biochem. J. 121:561–562; 1971.
3. Walsh, D.; Perkins, J.; Krebs, E. An adenosine 3′,5′-cyclic monophosphate-dependent protein kinase from rabbit skeletal muscle. J. Biol. Chem. 243:3763–3774; 1968.
4. Gilman. A. A protein binding assay for adenosine 3′,5′-cyclic monophosphate. Proc. Natl. Acad. Sci. USA 67:305–312; 1970.

Histamine-Induced Cyclic AMP Accumulation in Lungs of Pertussis-Vaccinated Mice

R. A. Ortez

ABSTRACT

Immunohistochemical procedures were used to locate the pool of cAMP that accumulates in normal and pertussis-vaccinated mice following either histamine or epinephrine challenge. These procedures showed that all major pulmonary compartments (i.e., vasculature, airways, and interstitium) contain some immunoreactive cAMP material. However, in vivo epinephrine or histamine challenge increased the cAMP content only in the vasculature. No effect of either agent was detected in bronchiole smooth muscle or interstitial tissue. The increased cAMP accumulation was observed in both normal and pertussis-vaccinated mice following epinephrine challenge, but only in pertussis-vaccinated mice after histamine challenge. An approximate 200% increase over basal levels was detected under both stimulus conditions. Measurements of changes in cAMP content by this immunohistochemical procedure agreed well with those determined by radioimmunossay of cell free homogenates. Pertussis-vaccinated mice were also shown to be hypotensive, displaying a 40% reduction in basal blood pressure. These two observations are combined with other pertinent findings to develop a hypothesis for the biochemical mechanism underlying increased histamine-mediated cAMP accumulation in pertussis-vaccinated mice.

INTRODUCTION

Bordetella pertussis-vaccinated mice have heightened susceptibility to a wide variety of pharmacological and immunological agents (1-4). While the biochemical basis for this hypersensitivity is unknown, similar changes have been observed in propranolol and dichloroisoproteranol-treated animals (5,6). Such observations were instrumental in the formulation of the β-adrenergic theory of bronchial asthma and led to the suggestion that the pertussis-vaccinated mouse might be a useful animal model for that human disease (7,8). The theory predicted that the levels of cyclic adenosine 3′,5′-monophosphate (cAMP) would be depressed in pertussis-vaccinated mice. However, determination of the cAMP content of several organs from normal and pertussis-vaccinated mice demonstrated an organ-specific histamine-mediated increase in cAMP in lungs of pertussis-vaccinated mice (9,10).

Studies involving pretreatment of pertussis-vaccinated mice with a variety of pharmacologic agents demonstrated that histamine-mediated cAMP accumulation is inhibited by tripelennamine (an H_1 receptor antagonist) and propranolol (a β-adrenergic receptor antagonist) (11,12). In addition a close temporal correlation was observed between the development of this cAMP abnormality and an inflammatory lesion in the lungs of pertussis-vaccinated mice (12-14). Therefore it was postulated that the increased cAMP response was associated with a protective phenomenon in histamine-challenged pertussis-vaccinated animals (12-14). Further clarification required determining the cell type accumulating cAMP in response to histamine or epinephrine.

We recently undertook studies to localize by immunohistochemistry the cAMP pools accumulating in pertussis-vaccinated mice (15,16). The results of these experiments including blood pressure measurements are the subject of this report. A model for histamine action in this system is described.

MATERIALS AND METHODS

Animals, Vaccination, and Challenge

Outbred female Swiss mice (22-26g) of the CFW and ICR strains, obtained from Charles River Breeding Laboratory, Wilmington, Massachusetts, and Sprague Dawley, Madison, Wisconsin, respectively, were housed in groups of six to eight and provided food and water ad libitum. Vaccination was accomplished by a single intraperitoneal (i.p.) injection of 10^{10} whole killed *B. pertussis* cells in 0.25 ml of physiological saline 5 days before sacrifice. Epinephrine (10 mg/kg) and histamine (3.6 mg/kg, histamine base as the diphosphate salt)

were administered i.p. in a volume of 0.025 ml/g of animal weight 1 minute before sacrifice. Control animals received comparable volumes of physiological saline. All drugs were prepared fresh from solid reagents the day of the experiment.

Tissue Preparation

After sacrifice by cervical dislocation the abdominal and thoracic cavities were opened, the trachea located and cannulated, and a volume (0.1 ml/7 g body weight) of optimum cutting temperature (OCT) compound (diluted 1:10 with physiological saline) was injected slowly to inflate the lung (17). The trachea was tied off and the lungs and heart removed. The left lung was cut free after ligation to prevent leakage of the OCT compound. This isolated, inflated lung was placed in an aluminum boat filled with undiluted OCT compound and frozen in a dry ice and methanol bath. The whole process required approximately 5 minutes from sacrifice to freezing. Tissues were then mounted on a cryostat microtome and 6μ sections obtained. After transfer to microscope slides and air-drying, the tissue sections were placed in a 68° C oven for an hour.

Cyclic AMP Immunocytochemistry

The procedure for staining and examining the tissues was modified from that described by Steiner et al. (18). All fluids (antisera, buffer, etc.) making contact with the tissue were adjusted to pH 6.1 ± 0.1. This was required to facilitate adhesion of the tissue to the slide. Increasing the pH above 6.5 led to a progressive detachment of the tissue from the slide. Following a 5 minute wash in 50 mM sodium phosphate buffer containing 15.4 mM NaCl (staining buffer), the tissues were covered with 20 μl of *first* antibody (50% ammonium sulfate fractions of goat anti-cAMP serum or normal goat serum) appropriately diluted in 20 mM 2-morpholinoethanesulfonic acid (MES) buffer (pH 6.1) and left overnight at room temperature in a humidified chamber. The next morning the slides were washed in three changes of staining buffer and the *second* antibody (fluoresceinated anti-goat IgG serum) was applied in a volume of 20 μl at a dilution of 1:40 in phosphate buffered saline (50 mM sodium phosphate, 154 mM sodium chloride, pH 6.1) and returned to the humidified chamber. One hour later the slides were removed and washed free of antiserum by three successive changes of staining buffer. The tissues were covered with 20 μl of 50% glycerin in staining buffer and a cover slip was applied. The sections were examined on a Leitz Dialux fluorescence microscope. Random areas on each slide were selected and photographed (Kodak Tri–X Pan) using a constant exposure of 30 seconds.

Quantitation of Immunocytochemistry

Assuming proportionality between the concentration of antigen (cAMP) and antibody (anti-cAMP serum) and the degree of staining observed, changes in cAMP were quantified by determining the maximum dilution of anti-cAMP serum still producing detectable staining. This dilution was termed the maximal effective dilution (MED) and could be averaged for several animals to yield mean maximal effective dilution (MMED), which could be used for comparison between groups. This is equivalent to determining the end point when determining the titer immunoglobulin by a standard precipitin procedure (19).

Figure 1 shows the results of a typical experiment to determine MED values. Following i.p. injections of two mice with either saline or epinephrine (10 mg/kg), the lungs were removed, embedded in OCT compound, and sectioned as described earlier. The sections were then reacted with varying dilutions of anti-cAMP serum ranging from 1:400 to 1:6400. A control slide was reacted with buffer only. All other parameters of the immunocytochemical procedure of cAMP localization were held constant as described earlier. After photographing typical regions of each slide, the photographic prints were arranged in order of increasing anti-cAMP serum concentration and the reciprocal of the highest dilution yielding detectable staining over the control slide taken as the MED. In the experiment shown in Figure 1 the MED values for the saline and epinephrine injected animals were 400 and 3,200 respectively for pulmonary vasculature. The averaging of MED values for several saline or epinephrine injected mice would yield an MMED value for each group.

Although this procedure is not actually quantitative for cAMP, it does allow quantitative comparisons between groups. For example, a change in the MED from 400 for control animals to 3,200 for hormone treated animals represents a 700% increase in cAMP content.
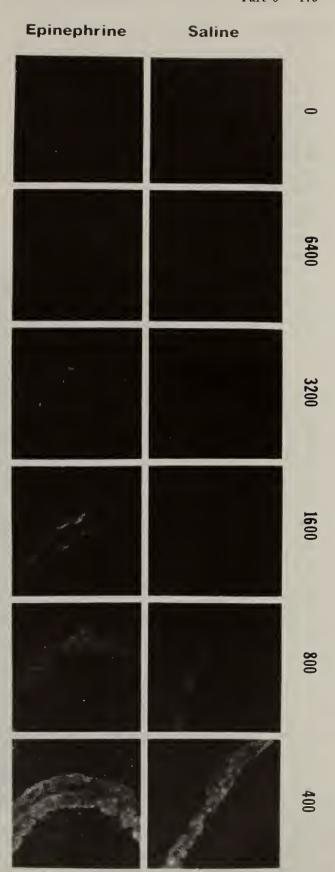
Cyclic AMP Extraction and Measurement

Frozen lung samples were homogenized in 4.0 ml of buffer (50 mM potassium acetate, 1 mM ethylenediaminetetraacetate, pH 7.0) for 5 seconds on a

Brinkman Polytron at a setting of 6. Immediately after homogenization 0.9 ml aliquots were transferred in duplicate to tubes containing 0.1 ml of 50% trichloroacetic acid. After clarification by centrifugation and neutralization by ether extraction, the samples were applied to 0.6 × 3 cm Dowex-1-formate ion exchange columns equilibrated with 0.1 N formic acid. After sample application the columns were washed with 6 ml of 0.1 N formic acid and cAMP was eluted with 6 ml of 2 N formic acid (20). The cAMP fractions were lyophilized and reconstituted in an appropriate volume of distilled water. Cyclic AMP recovery, as determined by addition of ^{3}H-cAMP to the homogenates, averaged 70–80%. Cyclic AMP was measured by a modification of the radioimmunoassay described by Steiner et al. (21) that has been described more fully elsewhere (12). Protein was determined on sodium hydroxide solubilized aliquots of homogenate by the method of Lowry et al. (22).

Blood Pressure Determination

Mice were anesthetized (45 mg/kg sodium pentobarbitol i.p.), the abdominal cavity was opened, and the dorsal aorta was located just anterior to the kidneys and teased free. After double clamps were placed on the artery, a catheter of PE 10 tubing was inserted between the clamps pointing in an anterior direction and ligated into place. This catheter was connected to a Beckman type RM dynograph recorder through a Statham P23DB pressure transducer. After the anterior clamp was removed blood pressure could be continously monitored for 1–2 hours with no noticable deterioration. Drugs were injected intravenously (i.v.) into the vena cava with a Hamilton syringe equipped with a 26-gauge needle. With care, three or four such injections could be administered before noticable deterioration occurred.

Figure 1. Composite photograph of serial sections of lungs from a saline and an epinephrine (10 mg/kg) challenged nonvaccinated mouse stained to determine MED values. The sections were reacted overnight with 1:400, 1:800, 1:1600, 1:3200 and 1:6400 dilutions of anti-cAMP serum. The "O" frames were sections reacted with diluent buffer only. The remainder of the anti-cAMP immunocytochemical and photographic procedures were held constant for all slides as described in the Methods section. The reciprocal of the serum dilution at which a fluorescent image first appears is taken as the MED value for that tissue. The MED values for these tissues were recorded as 400 and 3,200 respectively for the saline and epinephrine-treated mice. [Reprinted with permission from J. Cyclic Nucleotide Res. 4:233–244; 1978. Further reproduction prohibited without permission of copyright holder.]

Reagents

Pertussis vaccine was a gift from Eli Lilly and Co., Indianapolis, Indiana. Epinephrine hydrochloride and histamine diphosphate were purchased from Sigma Chemical Co., St. Louis, Missouri. OCT compound, a product of Miles Laboratories, was purchased from Scientific Products, and 2-N-Morpholine-ethanesulfonic acid (MES) buffer from Nutritional Biochemicals Corp., Cleveland, Ohio. Anti-cAMP serum was prepared in goats in collaboration with Bioteck Laboratories, St. Louis, Missouri, which also provided the normal goat serum. Fluoresceinated anti-goat IgG serum was purchased from Miles Biochemicals, Elkhart, Indiana.

RESULTS

Basal cAMP Distribution in Lung from Control and Pertussis-Vaccinated Mice

Cryostat sections from unchallenged control and pertussis-vaccinated mice were stained with normal goat serum (NGS) or goat anti-cAMP serum at a dilution of 1:400 overnight, then treated with fluoresceinated anti-goat IgG serum. This dilution of anti-cAMP serum yielded maximal staining in all lung compartments. Figure 2 shows that in an unchallenged, nonvaccinated animal all three major pulmonary compartments (i.e., vasculature, airways, and interstitium) gave positive reactions with anti-cAMP serum, suggesting the presence of cAMP.
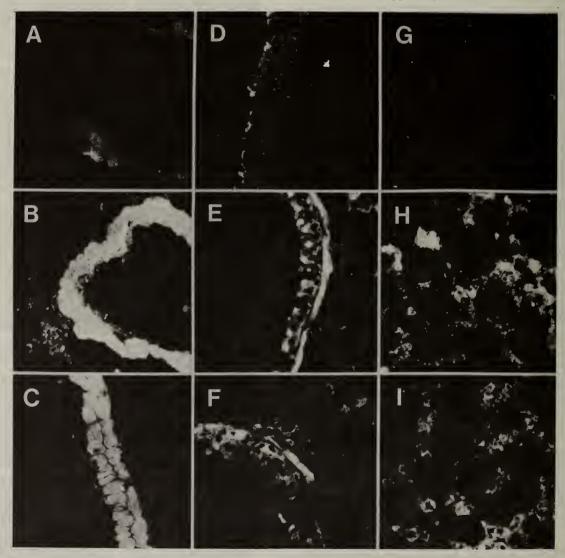
Figure 2. Photomicrographs (400×) of pulmonary blood vessels (A,B,C), broncheoli (D,E,F) and interstitium (G,H,I) from control animals stained with comparable dilutions (1:400) of normal goat serum (A,D,G); anti-cAMP serum (B,E,H); and enriched anti-cAMP antibody obtained from anti-cAMP serum (C,F,I) as first antibody. All sections were stained with fluoresceinated anti-goat IgG serum (1:40 dilution) as second antibody. [Reprinted by permission from J. Cyclic Nucleotide Res. 4:233–244; 1978. Further reproduction prohibited without permission of copyright holder.]

The most intensely staining area was the vascular musculature. Small artery walls from slides reacted with either NGS or anti-cAMP serum are shown in panels A and B respectively (Fig. 2). Panel C is a blood vessel from a slide stained with a more purified anti-cAMP antibody precipitated from whole serum with a cAMP-keyhole limpet hemocyanin complex. Panels D and E show bronchiole walls from NGS and anti-cAMP serum stained slides respectively. It is clear that a major cAMP positive area is the smooth musculature of the airways. Panel H (anti-cAMP stained) shows that the cytoplasm of many cells in the interstitial area stained for cAMP. This panel is in contrast to panel G, the NGS control, which shows no staining. Panels F and I show bronchiole wall and interstitium, respectively, which were stained with the more purified anti-cAMP antibody preparation. These staining patterns were identified for normal and pertussis-vaccinated mice.

Cyclic AMP Distribution in Lung Tissue from Control and Pertussis-Vaccinated Mice After in vivo Epinephrine or Histamine Challenge

In these studies sections of lung from mice challenged in vivo with epinephrine (10 mg/kg), histamine (3.6 mg/kg, histamine base as the diphosphate salt), or saline were stained with serial dilutions of anti-cAMP serum. The MMED values for each of three pulmonary compartments are presented in Table 1. From these data, it is clear that the vascu-

lature is the only tissue compartment that responds with increased cAMP accumulation to an in vivo challenge with epinephrine. The MMED value for this region increased from $1,534 \pm 392$ for control animals to $4,800 \pm 715$ for epinephrine-treated mice. This change in dilution represents an approximate 200% increase in cAMP content resulting from epinephrine challenge. Comparable MMED value changes ($1,734 \pm 321$ to $5,066 \pm 868$) were observed for the vasculature for saline and epinephrine-injected pertussis-vaccinated mice. In both cases the increased MMED values observed for sections from epinephrine treated mice were statistically significant ($p < 0.05$) from controls. The only other statistically significant change in MMED values was the 200% increase in cAMP in the vasculature of histamine-challenged pertussis-vaccinated mice (MMED $= 2,800 \pm 819$), compared to histamine challenged nonvaccinated mice (MMED $= 800 \pm 178$).

Relationship Between Immunocytochemical Staining and cAMP Content

In an attempt to compare cAMP content and immunofluorescence staining, animals were given i.p. injections of either propranolol (10 mg/kg) 30 minutes before sacrifice or epinephrine (0.5, 5.0, or 50.0 mg/kg) 1 minute before sacrifice. Control animals received comparable volumes of physiological saline. The animals were then killed, and the lungs were removed after being inflated with OCT compound. The portion of the lung used for tissue sectioning and immunocytochemistry was processed as described earlier. The remainder of the lung was rapidly frozen by compression between blocks of dry ice and processed to measure cAMP. Table 2 shows the MMED values for each experimental group along with the cAMP content as determined by radioimmunoassay. Good agreement exists be-

Table 1. MMED Values for Normal and Pertussis-Vaccinated Lung Following Saline, Epinephrine, or Histamine Challenge

Tissue and Treatment	Normal	Pertussis-Vaccinated
Saline [a]		
Bronchiole	$1,333 \pm 169$[d]	$1,008 \pm 199$
Interstitium	800 ± 178	$1,008 \pm 199$
Vasculature	$1,534 \pm 392$	$1,737 \pm 321$
Epinephrine [b]		
Bronchiole	$1,333 \pm 66$	$1,266 \pm 247$
Interstitium	733 ± 66	$1,008 \pm 199$
Vasculature	$4,800 \pm 715$[e]	$5,066 \pm 868$[e]
Histamine [c]		
Bronchiole	$1,060 \pm 168$	$1,600 \pm 358$
Interstitium	800 ± 178	$1,008 \pm 199$
Vasculature	800 ± 178	$2,800 \pm 819$[f]

[a] 0.1 ml/10g mouse weight i.p. one minute before sacrifice
[b] 10 mg/kg i.p. one minute before sacrifice
[c] 3.6 mg/kg base i.p. one minute before sacrifice
[d] MMED $\pm$ S.E. (n = 6)
[e] Statistically significant from saline control ($p < 0.05$)
[f] Statistically significant from control ($p < 0.05$)

Table 2. Effects of Propranolol or Epinephrine on Whole Lung Cyclic AMP Content and MMED Value for Lung Vasculature

	MMED[a]	cAMP[b]
Control	$2,857 \pm 921$	56.3 ± 9.1
10 mg/kg Propranolol	857 ± 136	47.6 ± 4.3
0.5 mg/kg Epinephrine	$5,714 \pm 1390$[c]	100.1 ± 33.1
5.0 mg/kg Epinephrine	$10,514 \pm 1516$[c]	197.6 ± 14.6[c]
50.0 mg/kg Epinephrine	$9,600 \pm 1431$[c]	253.6 ± 39.6[c]

[a] Mean Maximal Effective Dilution of Antisera $\pm$ SE (n = 6)
[b] pmoles/mg protein $\pm$ SE in whole lung (n = 6)
[c] Statistically significant from control ($p < 0.05$)

tween the MMED values from immunocytochemistry and the concentrations of cAMP. Propranolol depressed cAMP content as determined by either immunocytochemistry or radioimmunoassay, while epinephrine caused increases in cAMP content as determined by either procedure. Statistically significant changes from control ($p < 0.05$) were seen with both procedures at 5 and 50 mg/kg epinephrine.

Blood Pressure Alterations

Blood pressures were measured on groups of control mice and pertussis-vaccinated mice 6–7 days after vaccination. After an initial reading, each animal received an i.v. injection of 5 μg/kg epinephrine while the blood pressure was continuously monitored and recorded. After the blood pressure returned to preinjection levels each animal received an i.v. injection of 5 mg/kg histamine diphosphate, and blood pressure changes were recorded. When the blood pressure again returned to preinjection levels each animal received a second i.v. injection of 5 μg/kg epinephrine to reassess vascular responsiveness. Figure 3 shows a pair of representative tracings that demonstrate the integrity of the epinephrine pressor response throughout the experiment.

Table 3 records the mean systolic/diastolic pressure of normal and pertussis-vaccinated mice in response to the above injection protocol. From these data it is apparent that the major difference between normal and pertussis-vaccinated mice is that in the latter group basal blood pressure is reduced by approximately 40% relative to control animals. Both groups responded to epinephrine by approximately doubling their blood pressures relative to their respective basal levels. This was true for both epinephrine injections. Unlike the re-
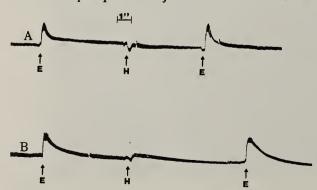
Figure 3. Effects of 5μg/kg epinephrine (E) and 5mg/kg histamine (H) on normal (A) and pertussis-vaccinated (B) mouse blood pressure.

Table 3. Effect of Epinephrine and Histamine on Normal and Pertussis-Vaccinated Mouse Blood Pressure

	Basal	Epi.[a]	Hist.[b]	Epi.[a]
Normal	86/69[c]	153/119	74/56	148/108
Pertussis	55/43	119/96	64/54	111/100

[a] 5μg/kg epinephrine i.v.
[b] 5mg/kg histamine diphosphate i.v.
[c] Systolic/diastolic (mean of 4 animals/group)

sponse to epinephrine, a difference between the two groups is suggested in their responses to histamine injection. Control animals showed a modest hypotensive response while pertussis-vaccinated mice showed an equally modest hypertensive response. However, neither response was statistically significant relative to basal levels.

DISCUSSION

A constant problem in measuring whole organ cAMP levels has been the frequent inability to determine the cell type(s) responsible for observed changes. This is well exemplified by previous studies in which it was demonstrated that histamine challenge in pertussis-vaccinated mice led to an increased pulmonary accumulation of cAMP (9–12). Unfortunately, at least three cell types seemed responsible (vascular smooth muscle, bronchiole smooth muscle, and polymorphonuclear leucocytes), and the interpretation of the physiological significance of the cAMP accumulation depended upon knowing which ones were responsible. The current study, using immunocytochemistry to localize cAMP, demonstrates that it is vascular smooth muscle that accumulates cAMP in response to epinephrine challenge. In the pertussis-vaccinated animal histamine also caused an increase in vascular cAMP content. It is noteworthy that the polymorphonuclear leucocytes, shown to accumulate in lungs of pertussis-vaccinated mice (13,14) did not accumulate additional cAMP as a result of either epinephrine or histamine challenge. However, this does not exclude their indirect involvement in cAMP accumulation.

It is important to realize that both histamine and epinephrine affected the same pulmonary compartments. This finding strengthens speculations concerning the interrelationship between histamine and epinephrine in this system, namely that histamine action is mediated through epinephrine and that the vasculature, not the airways, constitutes the

major target tissue. It is of great significance that histamine was effective in increasing pulmonary staining only in pertussis-vaccinated mice. This was to be expected for the dose of histamine used (10) and suggests that at this dose epinephrine release is induced in vaccinated but not control animals.

The observation that bronchiole smooth muscle levels of cAMP are unchanged after epinephrine or histamine challenge does not support a β-adrenergic blockade theory, at the level of cAMP production, in this system. Such a blockade would predict decreased levels of cAMP in this compartment under conditions of pertussis-vaccination and histamine challenge. However, the possibility that the β-adrenergic response might be interrupted at some point subsequent to the elevation of cAMP still exists. For example, cAMP-dependent protein kinase or requisite phosphate acceptor proteins may be deficient.

The studies dealing with in vivo epinephrine or propranolol administration (Table 2) demonstrate a positive correlation between whole lung cAMP content derived from radioimmunoassay and the MMED values derived from fluorescent staining. Propranolol was shown to reduce, and epinephrine to increase, whole lung cAMP content. To this extent, the utility of the antibody dilution technique for determining quantitative differences in immunocytochemically determined cAMP is supported.

Knowing the cellular location of the epinephrine- or histamine-mediated increase in pulmonary cAMP makes it possible to propose the following hypothesis for pertussis-induced histamine hypersensitivity for cAMP production. The hypotensive action of histamine produces a fall in systemic blood pressure and triggers a compensatory pressor response involving epinephrine release. Since the pertussis-vaccinated mouse is already markedly hypotensive (Table 3), the dose of histamine required to exceed the threshold for stimulating epinephrine release would be less than in control animals. Once released, epinephrine acts on both α- and β-adrenergic receptors in vascular smooth muscle to facilitate both a pressor response (α-receptor-mediated) and increased cAMP production (β-receptor-mediated). The increased susceptibility to histamine-mediated cAMP production is therefore associated with the decrease in the amount of histamine needed to lower blood pressure enough to cause compensatory epinephrine release. This hypothesis is supported by the wealth of evidence showing that histamine acts indirectly through catecholamine to cause the pulmonary cAMP increase (9,10,12,13,24).

The hypothesis helps explain several observations, such as the organ specificity associated with histamine-mediated cAMP accumulation, and the apparent involvement of epinephrine when there is no increased sensitivity of epinephrine-stimulated cAMP production (12). The restriction of this phenomenon to the lung probably reflects the organ's greater vascularity. This is supported by the observation of a smaller histamine-mediated increase in cAMP accumulation in liver, another highly vascular organ (10). Similar increases in cAMP probably occur in other organs as well, but their high ratio of nonvascular to vascular tissue may mask the effect. It is clear that this hypothesis does not depend on a change in the epinephrine responsiveness of any tissue for cAMP production, but rather on a change in the sensitivity for epinephrine release.

The fact that histamine failed to produce a fall in blood pressure in pertussis-vaccinated mice does not contradict this hypothesis. In fact the rise observed might be an expected result of histamine-mediated epinephrine release. Clarification of this point would require detailed analysis of the dose dependent relationships between histamine and altered blood pressure, as well as determination of blood epinephrine concentrations.

The mechanism by which pertussis vaccine causes the initial hypotensive state is not known, but may be related to its ability to bring about increased vascular permeability (25). We have also shown evidence for increased vascular permeability in the form of leucocyte and edema fluid accumulation in lungs of pertussis-vaccinated mice (12–14).

None of this necessarily relates to the mechanism of histamine-induced death in these animals, which remains unknown. Although death may be related to the developing hypotensive state, direct evidence is lacking, and in fact pertussis-vaccinated mice have been shown to develop normal pressor responses to intravenous epinephrine. Much has been said about a possible mechanism of death involving pulmonary insufficiency due to β-adrenergic blockade. The studies reported here suggest that no such blockade exists at the level of bronchiole smooth muscle cAMP production, but blockade at some subsequent step is clearly possible. In fact preliminary studies in this laboratory indicate some loss of cAMP-dependent protein kinase activity in lungs of pertussis-vaccinated mice. There could also be a supersensitization of the α-adrenergic receptors

of bronchiole smooth muscle. The cause of death in histamine-challenged pertussis-vaccinated mice remains to be elucidated.

ACKNOWLEDGMENTS

REFERENCES

1. Parfentjev, A.; Goodline, M. A. Histamine shock in mice sensitized with *Hemophilus pertussis* vaccine. J. Pharmacol. Exp. Ther. 92:411–413; 1948.
2. Kind, L. S. The altered reactivity of mice after inoculation with *Bordetella pertussis* vaccine. Bacteriol. Rev. 22:173–182; 1958.
3. Munoz, J.; Bergman, R. K.; Histamine-sensitizing factors from microbial agents, with special reference to *Bordetella pertussis*. Bacteriol. Rev. 32:103–126; 1968.
4. Morse, S. I. Biologically active components and properties of *Bordetella pertussis*. Adv. Appl. Microbiol. 20:9–26; 1976.
5. Townley, R. G.; Trapani, I. L.; Szentivanyi, A. Sensitization to anaphylaxis and to some of its pharmacological mediators by blockade of the beta-adrenergic receptors. J. Allergy 39:177–197; 1967.
6. Szentivanyi, A.; Katsh, S.; Townley, R. G. Effect of pertussis sensitization and pharmacological beta-adrenergic blockade on the *in vitro* uptake of D-glucose-2-C 14 into glycogen of diaphragm and upon the formation of $C^{14}O_2$ from glucose-1-C^{14} by adipose tissue. J. Allergy 41:107; 1968.
7. Szentivanyi, A. The beta adrenergic theory of the atopic abnormality in bronchial asthma. J. Allergy 42:203–232; 1968.
8. Szentivanyi, A. Effect of bacterial products and adrenergic blocking agents on allergic reactions. Samter, M., ed. Immunologic diseases, II. Boston: Little, Brown and Co.; 1971:356–374.
9. Ortez, R. A. Histamine induced accumulation of lung cyclic adenosine 3′,5′-monophosphate in pertussis vaccinated mice. Fed. Proc. 33:586, 1974.
10. Ortez, R. A. Effect of histamine on lung cyclic AMP levels in normal and pertussis-vaccinated mice. Biochem. Pharmacol. 25:523–526; 1976.
11. Ortez, R. A. Relation between histamine induced lung cAMP accumulation and death in pertussis vaccinated mice. Fed. Proc. 35:609; 1976.
12. Ortez, R. A. Pharmacologic blockade of the effect of histamine on lung cyclic AMP levels in normal and pertussis-vaccinated mice. Biochem. Pharmacol. 26:529–533; 1977.
13. Ortez, R. A.; Cowden, W. L. Pertussis-induced pulmonary inflammation and its relation to cAMP metabolism. Fed. Proc. 36:1037; 1977.
14. Ortez, R. A. On the relationship between inflammation and altered cAMP metabolism in lungs of *B. pertussis*-vaccinated mice. Inflammation 3:1–6; 1978.
15. Ortez, R. A. Mechanism of histamine-induced cyclic AMP accumulation in lungs of pertussis-vaccinated mice. Fed. Proc. 37:391; 1978.
16. Ortez, R. A. A semi-quantitative method for cyclic nucleotide localization by immunocytochemistry and its application in determining the distribution of cyclic AMP in lungs of normal and pertussis-vaccinated mice following histamine or epinephrine challenge. J. Cyclic Nucleotide Res. 4:233–244; 1978.
17. Baker, J. R.; Rosenkrantz, H. Volumetric instillation of fixatives and inert substances into mouse lungs. Stain Technol. 51:107–113; 1976.
18. Steiner, A. L.; Ong, S.; Wedner, H. J. Cyclic nucleotide immunocytochemistry. Adv. Cyclic Nucleotide Res. 7:115–155; 1976.
19. Kwapinski, J. B. The precipitation test. Methods of serological research. New York: John Wiley and Sons, Inc.; 147–161; 1965.
20. Murad, F.; Manganiello, V.; Vaughan, M. A simple, sensitive protein-binding assay for guanosine 3′,5′-monophosphate. Proc. Natl. Acad. Sci. U.S.A. 68:736–739; 1971.
21. Steiner, A. L.; Parker, C. W.; Kipnis, D. M. Radioimmunoassay for cyclic nucleotides. J. Biol. Chem. 247:1106–1113; 1972.
22. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265–275; 1951.
23. Krzanowski, J. J.; Polson, J. B.; Szentivanyi, A. Theophylline attenuation of histamine-induced increases in pulmonary adenosine-3′,5′-cyclic monophosphate (cAMP). J. Allergy Clin. Immunol. 55:133; 1975.
24. Krzanowski, J. J.; Polson, J. B.; Szentivanyi, A. Pulmonary patterns of adenosine-3′,5′-cyclic monophosphate accumulations in response to adrenergic or histamine stimulation in *Bordetella pertussis*-sensitized mice. Biochem. Pharmacol. 25:1631–1637; 1976.
25. Munoz, J. J.; Bergman, R. K. *Bordetella pertussis*—immunological and other biological activities. Immunology Series, vol. 4. New York: Marcel Dekker; 1977: 1–235.

Structural Features and Separation of Some of the Biological Activities of the *Bordetella pertussis* Endotoxin by Chemical Fractionation

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ABSTRACT

Bordetella pertussis lipopolysaccharide, obtained by the phenol/water procedure, has a gross chemical structure different from lipopolysaccharides isolated from enterobacteria. It is made up of two different polysaccharides (PS 1 and PS 2) of low molecular weight and two different lipids (Lipid X and Lipid A), all of which were isolated after controlled chemical fractionation of the macromolecule. The lipids are differentiated by the fatty acids they contain: the unusual 2-methyl-3-hydroxy-decanoic and -tetradecanoic acids, present in Lipid X are absent from Lipid A. Polysaccharide 1 and Polysaccharide 2 are both bound to Lipid A, the former through a single molecule of nonphosphorylated KDO, and the latter through a single molecule of phosphorylated KDO. The point of attachment of Lipid X is unknown.

The lipopolysaccharide possesses all the usual biological activities of enterobacterial endotoxins. The four main regions mentioned above were examined for their biological actions. The nonspecific antibacterial, antiviral, and adjuvant activities of the lipopolysaccharide are carried by the lipid fractions. The minor Lipid X is toxic, pyrogenic, and gives a positive local Shwartzman reaction, but the major Lipid A is inactive in these three tests.

INTRODUCTION

A wide spectrum of biological activities can be produced by lipopolysaccharide (LPS) endotoxins from Gram-negative bacteria. Some of these activities are summarized in Table 1. Many of these, such as the production of fever, elicitation of the Shwartzman phenomenon, and depression of blood pressure leading to shock and death, limit the use of the endotoxins in human clinical practice despite the fact that other activities such as adjuvanticity, nonspecific protection against bacterial or viral infections, and antitumor activity, would be of great theoretical and practical interest.

Despite the interesting biological effects of the whole pertussis organism, which is often used as a good adjuvant and immunopotentiator (1), surprisingly little attention has been given to the structure and functions of its endotoxin as compared to those of enterobacteria.

MATERIALS AND METHODS

Our aim, using pertussis LPS as a model compound, was to investigate whether the typical activities of endotoxins can be dissociated from each other by physical or chemical manipulations. For this purpose pertussis organisms (strains L84 and 1414, both in phase I) were cultured by Dr. R. Donikian at the Institut Mérieux in Cohen and Wheeler's liquid medium. The endotoxin was extracted from freshly sedimented cells by the phenol-water procedure of Westphal and purified by repeated high speed centrifugation. About 10 mg of LPS per liter of culture medium were usually obtained.

Table 1. Biological Activities of Lipopolysaccharides

	Reference
Toxicity in mice	(2)
Depression of blood pressure	(3)
Shwartzman phenomenon (local and generalized)	(4)
Pyrogenicity	(5)
Hypothermia in mice	(6)
Leukopenia, followed by leukocytosis	(7)
Platelet aggregation	(8)
Complement activation	(9)
Mitogenicity	(10)
Adjuvant activity	(11)
Elicitation of nonspecific resistance to infection	(12)
Macrophage activation	(13)
Production of a tumor-necrotic factor	(14)
Interferon production	(15)
Prostaglandin synthesis	(16)

RESULTS

Analysis of the LPS by gel chromatography (Fig. 1) using an Ultrogel ACA 44 column eluted with a pH 8 borate buffer containing 1% SDS gave a single peak (carbohydrate) included in the gel. This LPS, which appears to be homogeneous by this criterion as well as by analytical ultracentrifugation, contains in its polysaccharide moiety (Fig. 2) L-glycero-D-manno-heptose, glucose, glucosamine, glucuronic acid, 3-deoxy-2-octulosonic acid (KDO), and at least one or two unidentified amino compounds. It also contains very small amounts of amino acids, but it is not known whether these are constitutive elements of the macromolecule. The lipid moiety contains phosphorylated glucosamine and fatty acids, essentially myristic and β-hydroxy-myristic acids, but also small amounts of unusual fatty acids. We shall return to this point later.

During chemical analysis of the LPS we observed
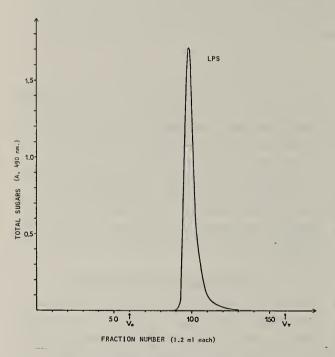
Figure 1. Elution pattern of the lipopolysaccharide from *B. pertussis* on Ultrogel ACA 44 column (1.4 × 127 cm). Eluent: 1 vol. of boric acid : sodium tetraborate 0.1 M buffer pH 8.8 + 9 vol. of 0.15 M NaCl + 10 g of sodium dodecylsulphate/l; final pH 8.0.
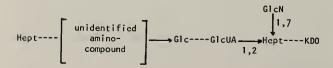
Figure 2. Substructures identified in the polysaccharide regions of *B. pertussis* lipopolysaccharide.

that this endotoxin contains two different molecules of KDO: nonphosphorylated KDO and KDO phosphorylated at position 5 (17). This finding is important, since it is known that in the LPS of enterobacteria the lipid moiety is linked to the polysaccharide part through the glycosidic bond of KDO, which is very acid labile. We observed further that the kinetics of the cleavage of the glycosidic bonds of the two KDO molecules in pertussis LPS were very different. Thus when the pertussis LPS was hydrolyzed with trifluoroacetic acid at pH 3 and 50° C the cleavage of the glycosidic bond of one KDO was complete after about 100 hours, as measured by the thiobarbiturate test (Fig. 3). The polysaccharide (polysaccharide I, PS 1) released during this hydrolysis was purified by dialysis and gel chromatography (18).

During the hydrolysis a precipitate was formed, from which we could extract with a mixture of toluene-methanol (1:1) a small amount of a complex lipid we called Lipid X. The residual material still contained an appreciable amount of neutral sugars; hence it is a glycolipid (GLP). We observed that polysaccharide 1 contained exclusively the non-
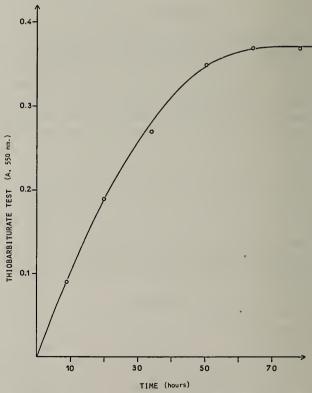

Figure 3. Kinetics of the release of Polysaccharide 1 from *B. pertussis* endotoxin treated with trifluoroacetic acid (pH 3, 50° C) as measured by the appearance of thiobarbiturate-positive material.

phosphorylated KDO as its terminal reducing sugar, while the glycolipid contained exclusively the phosphorylated KDO. The latter is not reducible; therefore the glycosidic bond of this phosphorylated KDO was not cleaved during hydrolysis with trifluoroacetic acid.

Hydrolysis of the glycolipid with 0.25 M HCl during 30 minutes at 100° C (Fig. 4) released a second polysaccharide (polysaccharide 2), which was purified like PS 1 by dialysis and gel chromatography. After this second hydrolysis a precipitate still-remained. We called it Lipid A by analogy with the precipitate obtained from enterobacterial endotoxins upon treatment with 0.1 M acetic acid.

The procedure outlined gave us two lipid fractions, Lipid X and Lipid A, containing only negligible amounts of neutral sugars, and two polysaccharides of lower molecular weight than polysaccharides obtained from wild strains of enterobacterial endotoxins. PS 1 is not phosphorylated, and its molecular weight as estimated from its KDO content is about 2,800. PS 2 is phosphorylated, and its molecular weight is about 3,600 when estimated by the same procedure. The two lipid fractions can also be distinguished by their chemical composition, since we observed by gas-liquid chromatography/mass spectrometry (Fig. 5) that two "unusual," branched chain fatty acids, namely 2-methyl-3-hydroxy-decanoic and 2-methyl-3-hydroxy-tetradecanoic acids (19) were present in Lipid X and were absent from Lipid A.

Biological Activities

These four fractions isolated from the pertussis endotoxin as well as the glycolipid and the LPS
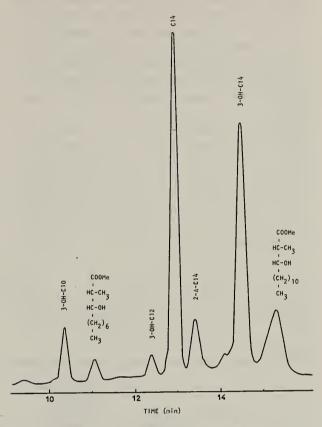
Figure 5. Separation by gas-liquid chromatography of the fatty acid methyl esters of Lipid X (from ref. 19).
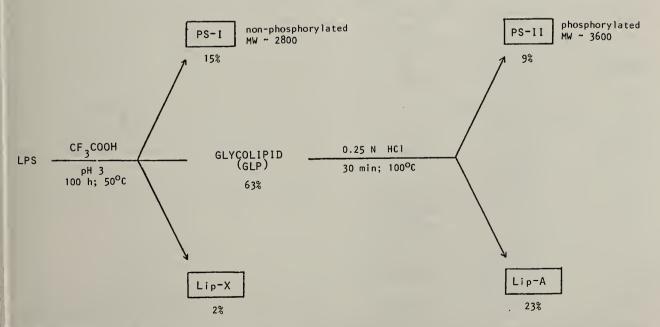
Figure 4. Fragmentation of the *B. pertussis* endotoxin.

were tested for several biological activities by Drs. Ayme, Mynard, and Roumiantzeff from the Institut Mérieux. Results obtained in five tests dealing with undesirable activities, such as general toxicity measured by the mouse weight-gain test, pyrogenicity, local Shwartzman reaction, local inflammation, and histamine sensitization, are summarized in Table 2. All fractions are negative in the histamine sensitization test, which proves that they were not contaminated with the histamine-sensitizing factor (HSF).

For other undesirable effects, the first line of Table 2 gives the "limit dose" [1] of the fractions below which the toxic effect is not perceptible: the glycolipid and Lipid X are toxic since the limit dose is low, whereas PS 1, PS 2, and Lipid A are nontoxic. Data obtained with formaldehyde treated *Corynebacterium parvum* cells used as a nontoxic immunopotentiator (20) are given for comparison.

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[1] The mean values of the weight change (Δp) observed 24 hours after injection of the material to be tested plotted against the log of the doses used gives a straight line: the "limit dose," for which Δp is zero, is obtained by extrapolation.

Analogous results were obtained for pyrogenicity and for the Shwartzman reaction.

Table 3 summarizes the results obtained with these fractions for antibacterial and antiviral activity and for adjuvant power for antibody production. It appears that the LPS and the glycolipid protect mice well against bacterial challenge; this activity is still present in the two lipid fractions but both polysaccharides are inactive. For adjuvant activity the most potent fractions are the toxic Lipid X and the nontoxic Lipid A. The antiviral activity of the LPS appears to reside in the two lipid fractions. The considerable difference observed in the antiviral activity for the EMC and SFV viruses is related to the challenging doses: 1.7 LD_{50} of EMC and 250 LD_{50} of SFV were used. Nevertheless it appears that the antiviral activity of the LPS is also carried by the Lipid A and Lipid X fractions.

CONCLUSION

The data presented establish that pertussis endotoxin has a general gross structure different from endotoxins isolated from enterobacteria: two dif-

Table 2. Undesirable Effects of the Endotoxin Fragments

Tests	Animals (injection)	Expression of the Results (time of observation)	LPS	PS–I	GLP	Lip–X	PS–II	Lip–A	Coryne-bacterium parvum (formolinized)
MWGT	10 mice OF 1 ♀ (25–400 μg i.p.)	limit dose D_0 μg (1 day)	<25	>400	25	124	>400	400	300
Pyrogenicity	3 New Zealand rabbits (2 μg/kg i.v.)	$\Sigma \Delta t$ °C (during 3 hours)	5.3	0.2	6.65	4.3	0	1.1	<1.15
Local Shwartzman phenomenon	New Zealand rabbit (25–400 μg i.d. + 20 hours later: LPS *Shigella* 100 μg i.v.)	necroses marked 0 to 4 Σ of 6 values (6 hours)	14.5	n.d.	6.5	5.5	0	0.5	0
Local inflammation	New Zealand rabbit (25–400 μg i.d.)	diameter of the inflammation (mm) (20 hours)	7	0	16.5	9	1	6	13
HSF	CFW mice (100 μg i.p. + 5 days later histamine 1/30 LD_{50} i.v.)	death (2 hours)	—	—	—	—	—	—	—

MWGT: Mouse weight gain test; HSF: Histamine sensitizing factor

Table 3. Immunopotentiation Activities of the Endotoxin Fragments

Tests	Mice (stimul. dose)	Delay	Cells Injected (dose)	Expression of the Results (time of observation)	LPS	GLP	PS-I	PS-II	Lip-A	Lip-X	Corynebacterium parvum	Control (Saline)
Anti-bacterial activity	10 OF1 ♀ (12.5; 50; 200 µg i.p.)	4 days	*Salmonella typhi* (11 LPD$_{50}$; i.p.)	Protective dose (PD$_{50}$) µg	44	10	>>200	>>200	137	37	>2500	
			Pseudomonas aeruginosa (8 LD$_{50}$; i.p.)	(4 days)	>200	110	>>200	>>200	>>200	170		
Antiviral activity	10 OF1 ♀ (200 µg i.p.)	24 hours	EMC (i.p.) (1.7 LD$_{50}$)	Surviving animals %	100	100		60	100	100	25	25
							n.d.					
			SFV (sc) (2 50 LD$_{50}$)	(11 days)	100	90		10	70	100		10
Adjuvant activity	8 OF1 ♀ (200 µg i.p.)	0	Valence A Human influenza vaccine (10 I U.; i.p.)	Increase of humoral antibodies (21 days)	2.4	2.7	1.8	2.6	4.6	5.1	3.5	1
			Valence B		2.7	2.7	2.3	4.3	3.7	6.7	3.2	1

EMC: Encephalomyocarditis virus; SFV: Semliki Forest Virus

ferent polysaccharides and two different lipids are present. Polysaccharide 2 is bound to Lipid A through a single molecule of phosphorylated KDO. Polysaccharide 1 probably is bound to Lipid A through a single molecule on nonphosphorylated KDO. The point of attachment of Lipid X is still unknown.

This lipopolysaccharide possesses all of the usual biological activities of enterobacterial endotoxins, but it has low pyrogen activity and is active in the microgram range, while enterobacterial endotoxins are pyrogenic at the nanogram level. After controlled chemical fractionation the four main regions mentioned above were isolated, as was glycolipid remaining after removal of Polysaccharide 1 and Lipid X from the intact endotoxin. Examination of the biological actions of these fragments revealed that the immunopotentiating activities of the endotoxin were due to the two lipid fractions. The minor Lipid X is toxic, pyrogenic and positive in the Shwartzman reaction, whereas the major Lipid A is nontoxic, nonpyrogenic, and gives a negative Shwartzman reaction.

REFERENCES

1. Dresser, D. W.; Phillips, J. M. The cellular targets for the action of adjuvants: T-adjuvants and B-adjuvants. Ciba Found. Symp. 18:3–28; 1973.
2. Ribi, E.; Milner, K. C.; Perrine, T. D. Endotoxic and antigenic fractions from the cell wall of *Salmonella en-teritidis*. Methods for separation and some biological activities. J. Immunol. 82:75–84; 1959.
3. Boquet, P.; Delaunay, A.; Lehoult, Y.; Lebrun, J. Action d'un antigène typhique purifié sur le système circulatoire périphérique du lapin. Ann. Inst. Pasteur 74:136–140; 1948.
4. Shwartzman, G. Studies on *Bacillus typhosus* toxic substances. I. Phenomenon of local skin reactivity to *B. typhosus* culture filtrate. J. Exp. Med. 48:247–268; 1928.
5. Greisman, S. E.; Hornick, R. B. Comparative pyrogenic reactivity of rabbit and man to bacterial endotoxin. Proc. Soc. Exp. Biol. Med. 131:1154–1158; 1969.
6. Prashker, D.: Wardlaw, A. C. Temperature responses of mice to *Escherichia coli* endotoxin. Br. J. Exp. Pathol. 52:36–46; 1971.
7. Thomas, L. The physiological disturbances produced by endotoxins. Ann. Rev. Physiol. 16:467–490; 1954.
8. Spielvogel, A. R. An ultrastructural study of the mechanisms of platelet-endotoxin interaction. J. Exp. Med. 126:235–249; 1967.
9. Gewurz, H.; Snyderman, R.; Mergenhagen, S. E.; Shin, H. S. Effects of endotoxic lipopolysaccharides on the complement system. Ajl, S.; Kadis, S.; Montie, T. C., eds. Microbial toxins, vol. 5. New York: Academic Press, Inc.; 1971:127–149.
10. Bona, D.; Damais, C.; Galanos, C.; Chedid, L. The mitogenic activity of lipopolysaccharides on lymphocytes in culture. Il. Comparative study in five mammalian species. Urbaschek, B.; Urbaschek, R.; Neter, E., eds. Gram-negative bacterial infections and mode of endotoxin reactions. New York: Springer-Verlag; 1975:182–189.
11. Johnson, A. G.; Gaines, S.; and Landy, M. Studies on the O antigen of *Salmonella typhosa*. V. Enhancement of antibody response to protein antigens by the purified lipopolysaccharide. J. Exp. Med. 103:225–246; 1956.
12. Howard, J. G.; Rowley, D.; and Wardlaw, A. C. Stimulation of non-specific immunity by the "Lipid A" component of bacterial lipopolysaccharide. Nature 179:314–315; 1957.
13. Alexander, P.; Evans, R. Endotoxin and double stranded

RNA render macrophages cytotoxic. Nature 232:76–78; 1971.

14. Carswell, E. A.; Old, L.J.; Kassel, R. L.; Green, S.; Fiore, N.; Williamson, B. An endotoxin-induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. U.S.A. 72:3666–3670; 1975.

15. Schiller, J. G.; Ribovich, R.; Feingold, D. S.; Youngner, J. A. Interferon production in mice by components of *Salmonella minnesota* R 595 Lipid A. Infect. Immun. 14:586–589; 1976.

16. Dey, P. K.; Feldberg, W.; Wendlandt, S. Lipid A and prostaglandin. J. Physiol. (Lond.) 239:102–P; 1974.

17. Chaby, R.; Szabo, L. 3-Deoxy-2-octulosonic acid 5-phosphate: a component of the endotoxin of *Bordetella pertussis*. Eur. J. Biochem. 59:277–280; 1975.

18. LeDur, A.; Caroff, M.; Chaby, R.; Szabo, L. A novel type of endotoxin structure present in *Bordetella pertussis*. Isolation of two different polysaccharides bound to lipid A. Eur. J. Biochem. 84:579–589; 1978.

19. Haeffner, N.; Chaby, R.; Szabo, L. Identification of 2-methyl-3-hydroxydecanoic and 2-methyl-3-hydroxy-tetradecanoic acids in the "Lipid X" fraction of the *Bordetella pertussis* endotoxin. Eur. J. Biochem. 77:535–544; 1977.

20. Halpern, B.; Fray, A.; Crepin, Y.; Platica, O.; Lorinet, A. M. Rabourdin, A; Sparros, L.; Isac, R. *Corynebacterium parvum*, a potent immunostimulant in experimental infections and in malignancies. Ciba Found. Symp. 18:217–233; 1973.

DISCUSSION OF PART 3

DR. LEHRER: I would like to ask Dr. Kong about his polyacrylamide gel electrophoresis. You stated that you observed only one band in your purified fraction. You used a 5% gel, and you said that the molecular weight of your substance was 73,000. I wanted to ask you why you chose 5% gel, because in polyacrylamide gel electrophoresis, you do not get much fractionation of a molecule that size in a 5% gel. You should probably use a 7 or 8% gel.

DR. KONG: We have tried other concentrations of gels. Five percent gel at pH 4.5 proved to be the most useful system, and we found only one band with the purified material. However, after treatment of the molecule with SDS and 2-ME we were able to detect four distinct polypeptides with molecular weights of 13,400, 17,400, 19,300, and 23,500.

DR. LEHRER: With the higher concentration of gels, do you just get one band without treating the molecule?

DR. KONG: Yes, at higher gel concentrations we do get one band with undissociated LPF.

DR. LEHRER: The other question I had was, how do you relate the mitogenic activity of LPF to its in vivo activity? Do you believe it is related?

DR. KONG: Lymphocytosis involves the redistribution of lymphocytes from the lymphoid tissues into the blood and is mainly due to the inability of lymphocytes to home at a normal rate back to the lymphoid tissue. I want to point out that in lymphocytosis we use much lower doses of LPF, which are further diluted in the blood. However, the mitogenic response in vitro requires much higher doses of LPF.

DR. LEHRER: So you believe the mitogenicity is not related to the lymphocytosis?

DR. KONG: We don't have any evidence that they are related. Furthermore, I want to stress one thing: we do not know whether, in fact, it is due to the same active site on the molecule.

DR. KLEIN: Your demonstration that your fraction has in vitro tumoricidal potential, I find most exciting. Do you have any information on the tumoricidal ability of this substance in vivo?

DR. KONG: We haven't tested the material in vivo for tumoricidal activity.

DR. KLEIN: I think the literature on the nonspecific adjuvant effect of pertussis regarding tumors is confusing at this point. It is interesting that you have this LPF component which conceivably has tumoricidal activity, and that the endotoxin component described by Dr. Chaby may also have tumoricidal properties.

DR. J. B. ROBBINS: Dr. Andersen and Dr. Kong, have you exchanged products to see that the materials you are working with are identical?

DR. ANDERSEN: Not yet, but we have been invited to Drs. Morse and Kong's laboratory on Tuesday, and I

hope something will come out of that.

DR. J. B. ROBBINS: Some of these activities may be mediated by the same mechanism, but the final reaction may differ.

DR. ANDERSON: I quite agree.

DR. STAINER: I would like to ask Dr. Lehrer a question. When you had your purified preparation, you stated that all the activity disappeared when you checked the individual electrophoretic fractions. Did you combine any of these fractions to see whether it could be a multicomponent thing, or did you just check each fraction?

DR. LEHRER: Well, that is a good point. We did not combine the fractions, but that is something that we want to do, to see if it is possible that we actually have, perhaps, subunits, because these polyacrylamide gels are run in the presence of 4M urea, so we could be disassociating subunits which are inactive. That is something that we want to do.

But we do know that after we purify this substance we lose activity, and it becomes extremely labile. Even the material that we put on the gel is not stable for a very long period of time. So it is my feeling that we probably have lost the activity during the process. But still it is something to try to recombine the different fractions.

DR. HELTING: I would also like to ask a question about the polyacrylamide gel separation. Have you tried pH 4.5 electrophoresis, where you could probably run your sample without urea and see whether you could recover the activity?

DR. LEHRER: Actually we have run it at a pH below that, a little over pH 3. The buffer that we use 4M urea in, I believe, is 0.9% acetic acid, so it is at a very low pH; and it just happens to be the system in which we have had some success in separating components and getting some mobility.

DR. HELTING: But you have not tried the Reisfeld system, which has been used for the LPF purified from this supernatant?

DR. LEHRER: No.

DR. MUNOZ: Could you tell us how you prepare your antiserum against the HSF?

DR. LEHRER: We use a lymph node immunization system. This is a procedure that has been developed for producing antisera to extremely small amounts of antigens. What one does is inject microgram amounts of antigen into the lymph nodes of rabbits. You need approximately 40 micrograms in your first dose. And then 4 weeks later, you immunize them with 100 micrograms in the foot pad. This is really a lovely method for producing antisera to small amounts of antigen, and we have had a lot of success with it, not only in terms of producing antisera to pertussis fractions, but also to other protein antigens. The only problem about the system is that you have to have a decent antigen to begin with. It is not going to make a poor antigen a good antigen, but if you have small amounts of an antigen, it works very well.

DR. BLASKETT: Dr. Lehrer, in your production of these antibodies, were you sure that your rabbits were free of *Bordetella bronchiseptica*? It is a common occurrence that rabbits are infected with *Bordetella bronchiseptica*, and you mentioned that your normal rabbit serum had a level of neutralizing activity for HSF. I wonder whether *B. bronchiseptica* has anything to do with that.

DR. LEHRER: Well, we haven't been able to find any neutralizing activity of normal rabbit serum when tested in vitro—in other words, if the serum is added to HSF, incubated, centrifuged, and then injected into mice and they are challenged with histamine 4 days later. The problems that we have had arise if the serum is administered after HSF injection, and we find that we get reduced histamine sensitizing activity. I don't think this is due to antibodies. Perhaps it may be in some sort of plasma extender activity.

DR. J. B. ROBBINS: Dr. Ui, one of the problems posed by the current usage of our whole cell bacterial vaccine is altered central nervous system function in a few individuals. This rare adverse reaction has been postulated to be due to altered carbohydrate metabolism, perhaps exactly the mechanism that you propose for your protein. Have you had a chance to use your measurement techniques with vaccines or in patients who have had adverse reactions to pertussis vaccine?

DR. UI: You mean insulin secretion or other carbohydrate metabolism?

DR. J. B. ROBBINS: Or the potentiation of, or the inhibition of adrenalin protection against insulin secretion.

DR. UI: Insulin is secreted only when it is stimulated, so in the basal state there is no trouble. In experimental animals I have found some altered carbohydrate metabolism. For example, there is a decrease in glycogen in liver, but I think this is due to the secretion of insulin.

DR. J. B. ROBBINS: Have you studied the interaction between your purified protein and other bacterial components that have surface activity, such as lipopolysaccharide?

DR. UI: I have not.

DR. HEWLETT: Over the past several years a number of other people and I have been particularly bothered by the suggestion that the pertussis organism induces a β-adrenergic blockade, and I see even in some of the slides that were presented here that it is still listed as one of the biological activities of the organism.

I think, first of all, it is unreasonable to claim receptor blockade on the basis of indirect effects, which may well be mediated by some mechanism distal to the receptor. Table I illustrates the data that were the basis for the

Table I. Effect of Pertussis Vaccine Sensitization on the Response of Serum Glucose to Epinephrine

| | Serum Glucose (mg/dl ± SE) | | |
	Basal	Post epinephrine	% Change
Control	100±6	251±3	+151
Pertussis sensitized	102±18	180±13	+76

original hypothesis of β-adrenergic blockade. When normal animals are given subcutaneous epinephrine, serum glucose increases approximately 100%. In pertussis-sensitized animals, with normal basal serum glucose, the response to epinephrine administration is markedly damped.

In an attempt to determine the status of the β-adrenergic receptor in pertussis-sensitized animals, we have measured receptor number and affinity by iodohydroxybenzylpindolol binding and receptor function by isoproterenol-stimulated adenylate cyclase in rat reticulocytes.[1] Table 2 summarizes the results. Under conditions in which the response of serum glucose to epinephrine was reduced, there was no alteration of receptor number or affinity or adenylate cyclase activity. In addition, as has been noted earlier, there was a sixfold increase in serum insulin. We feel that these data, in conjunction with previous available information, establish the fact that pertussis administration does not produce β-adrenergic blockade. In light of the beautiful study presented by Dr. Ui, I think it is now reasonable to assume that the effects on serum glucose can be explained on the basis of altered insulin secretion in the pertussis-treated animal.

We have also examined this problem further and have results which differ somewhat from those of Dr. Ui and his coworkers. We have used HSFS/N mice derived from the NIH line and bred specifically for sensitivity to the histamine sensitizing factor (HSF) of *B. pertussis*. When these animals are given Lot 7b pertussis vaccine (25 opu IV), there is a biphasic response which occurs over a 2-week period. Initially, there is a moderate hyperinsulinemia lasting less than 48 hours that is associated with significant hypoglycemia (Table 3). This effect appears to be mediated by endotoxin. Second, there is a sustained hyperinsulinemia of enormous proportions, peaking at 7 days after inoculation and lasting more than 14 days. This response, during which basal insulin levels reach more than 50 times normal, is associated with normal serum glucose and is mediated by a heat labile component of the bacterium. We presume that this represents a slightly different response to the same factor as that studied by Dr. Ui. His data indicated that animals treated with IAP manifested normal serum insulin levels, but responded to insulin secretagogues with enhanced secretion. Even when whole pertussis vaccine was used, his results were the same. We feel, therefore, that there are substantial species and strain differences in the responses to this bacterial product, as has been illustrated for other biological activities of the pertussis organism. Knowledge of the mechanisms involved in these varied responses will be of great help in understanding the differences in severity of clinical pertussis from patient to patient and the heterogeneity of responses to whole cell pertussis vaccine. I am interested in Dr. Ui's thoughts on this subject.

DR. UI: I understand that your results are quite different from mine in that the basal insulin secretion is also enhanced. But I think the differences may be due to the

[1] Hewlett, E.; Spiegel, A.; Wolff, J.; Aurbach, G.; and Manclark, C. R. *Bordetella pertussis* does not induce β-adrenergic blockade. Infect. Immun. 22:430–434, 1978.

Table 2. Summary of Effects of Pertussis Vaccine on the Rat

Parameter [a]	Effect
Serum: Glucose	None
Glucose after epinephrine	Reduced 46%
Insulin	Increased sixfold
Reticulocyte: β-Adrenergic receptor number	None
β-Adrenergic receptor affinity	None
Isoproterenol-stimulated adenylate cyclase	None

[a] Relative to unvaccinated control

difference in the strain of the rat—I mean, in some strains the liver glycogen is still high, even in the fasted state. I used the Wistar-derived strain (i.e., the Donryn strain). In that strain we have very low glycogen levels in the liver in the fasted state. So I think that if you inject the vaccine into your rat, glycogen is decomposed in the liver, and the glucose produced may act as a stimulus for insulin secretion.

DR. MUNOZ: I think that I should point out that Dr. Hewlett's work was done in mice, and that there is a marked difference in the response of rats and mice with respect to induction of insulinemia.

DR. HEWLETT: This is true, except that in the original studies done by Gulbenkian, it was shown that both rats and mice responded to whole pertussis vaccine with hyperinsulinemia. It was more dramatic in the mouse, but both species had a measurable basal hyperinsulinemia.

DR. MUNOZ: Which also emphasizes the importance of strain differences.

DR. LEHRER: I just want to mention that we tried to look for a β-blocking activity in our partially purified extracts of HSF, using a pigeon erythrocytes system in collaboration with Arnold Roho in John Singer's lab in La Jolla. What they do is use β-stimulating agents with the pigeon erythrocytes, and then measure increases in cyclic AMP. They found that none of our extracts could inhibit the stimulation.

DR. J.B. ROBBINS: I am still seeing whether, in these two systems studied by Dr. Hewlett and Dr. Ui, we can find some way of predicting why some children get these

Table 3. Effect of Pertussis Vaccine on Serum Glucose and Insulin in the HSFS/N Mouse

Time After Vaccination	Serum Glucose [a] (mg/dl)	Serum Insulin [b] (μU/ml)
8 hours	60±5	320±60
7 days	115±7	1490±300

[a] Before vaccination 120±16
[b] Before vaccination 20±10

adverse reactions to whole bacterial cell vaccines. Now LPF, in addition to its many other actions, alters vasomotor reactivity in the intact animal. Is it possible that in some of these experimental situations the CSF glucose varies under these conditions?

DR. UI: I haven't tried it.

DR. MUNOZ: I should point out that we also have some evidence that the β-receptors are not blocked. As I indicated in my talk, in the parotid gland studies employing incorporation of radioactive thymidine, we could not get any evidence that pertussigen had blocked the β-receptor, while endotoxin did.

DR. BLASKETT: May I ask Dr. Zakharova to tell us again about the heat stability of her enzyme? I am not quite sure whether she said it was stable to 56 or 50°C.

The other thing I would like her to comment on: She gave a table of enzyme activities of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*. As I have them, *B. pertussis* was 320 units, *B. parapertussis* was either 0 or 8, and *B. bronchiseptica* was 34. The 34 with *B. bronchiseptica* rather intrigues me. Is it significantly different from the *B. parapertussis* figure and would it suggest that *B. bronchiseptica* has a little of what *B. pertussis* has?

DR. ZAKHAROVA: We have determined the difference between the three species with respect to the activity of this enzyme. But I don't know why they differ. The enzyme was inactivated at 50°C.

This enzyme is very stable in comparison with intracellular adenylate cyclase. We know that this enzyme is stable at 50°C.

DR. HEWLETT: You measured the stability while it is still inside the cell? Is that what you mean?

DR. ZAKHAROVA: It is just a crude enzyme.

DR. HEWLETT: Just a crude enzyme in the supernatant. All right.

If I can respond briefly to this question about *B. parapertussis* and *B. bronchiseptica*, we have looked at all those strains and find that there is a difference between *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* with decreasing cyclase activities as you go through the three of them, but there are substantial amounts of extracytoplasmic adenylate cyclase in all three. We have not done the studies enough times to know whether the differences are significant. Certainly these three are all lumped together in comparison to all other bacteria we have looked at that have no extracellular adenylate cyclase.

DR. GOTTSCHLICH: As I understand the work presented yesterday, while I am not completely clear on some details that you presented, there seems to be an activation of this enzyme by some factor obtainable from red blood cells. I was just wondering, what are the plausible models that are being worked on at this point concerning this activation? What is going on in this field?

DR. ZAKHAROVA: We have not studied the activation of the adenylate cyclase.

DR. HEWLETT: There are a number of details of the activation process that we didn't go into yesterday for lack of time. It is apparent from the loss of activatability of the enzyme as it is purified that probably some other component is required in the activation process. The actual mechanism we do not know. When we look at

the solubilized enzyme, which still responds with a seven-fold increase in activity, it is apparent that the effect is completely one on V-max of the enzyme, and no effect on Km for ATP. There are no data yet to determine whether there is a binding of the activator to the enzyme molecule itself while it is associated with the whole organism, and the additional data that we have indicate that it is not a simple proteolysis process which has been seen in a number of mammalian adenylate cyclases.

DR. WOLFF: I think one should keep in mind that the current model for mammalian cyclases is one of a catalytic unit and a receptor, with two regulatory proteins between the receptor and the catalytic unit: one which binds G nucleotides and another which binds fluoride. Our plan is to see whether this activator can, in some way, mimic the regulatory units of mammalian cyclases.

DR. SATO: Dr. Munoz, what substances are released in the brain when *B. pertussis* cells are injected in the brain of an immunized animal?

DR. MUNOZ: It is known that antigen-antibody reactions in vivo activate many enzymes. Complement is one of a series of enzymes that are activated. When this happens, a number of vasoactive substances are produced. Histamine is only one of them. Serotonin, a slow reacting substance, and many active peptides are also known to be released during antigen-antibody reactions, or by sensitized cells reacting with antigen.

DR. SATO: Is it a specific response?

DR. MUNOZ: The factors released are not specific. That is why I mentioned the comparison between the allergic encephalomyelitis model and the mouse-protection test.

DR. SPEIRS: This conference has emphasized the variety of physiological and immunological actions that various components of pertussis vaccine may have. While its adjuvant effect on T-dependent antigens is well known, its effect on T-independent antigens has not been so thoroughly studied. We have been immunizing mice by s.c. or i.p. injections of type III pneumococcal polysaccharides (S3) given as a single antigen or mixed with DTP (a vaccine containing diphtheria and tetanus toxoids plus pertussis vaccine). Although the s.c. injections gave essentially the same results whether the S3 was given singly or in a mixed vaccine (Fig. 1), there were marked differences in the i.p. injections. Intraperitoneal injection of the antigenic mix appeared to suppress S3 antibody formation on day 5, the peak period of IgM antibody formation.

In order to determine if this suppressive action was directed at the S3 antigen or at the host responses, the S3 was injected separately from the DTP. High S3 antibody responses occurred only when the concurrent DTP injections were given s.c. (Fig. 2). Intraperitoneal injection of DTP suppressed the responses regardless of whether the S3 was given s.c. or i.p.

We, of course, suspected the PV component of DTP as having the suppressive action when given i.p. Since it did not have to be mixed with S3 in order to produce its suppressive action, we set up an experiment in which PV or DTP was given at various times prior to or following an s.c. injection of S3. When PV or DTP was given i.p. on or about the time of an s.c. injection of
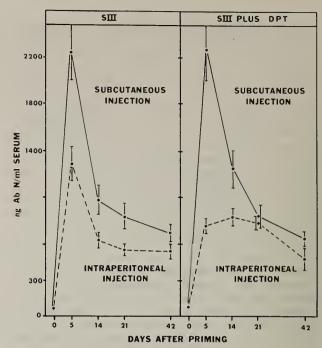
Figure 1. Antibody responses to SIII and SIII plus DTP comparison of subcutaneous and intraperitoneal injections.

S3, there was a marked suppression of the S3 antibody response (Fig. 3). However, if the PV or DTP was given 2 weeks prior to, or 2 or 3 days following the S3 injection, there was an augmentation of the S3 antibody titers. Each line of the chart illustrates the antibody titers obtained by pooling serum samples from five mice.

The data clearly demonstrate that the action of PV upon S3, a T-independent antigen, depends on the timing of the two injections in relation to each other and also upon the site of PV injection. These results are quite different from the reported effects of pertussis vaccine upon T-dependent antigens and serve as an example of the wide variety of effects these particular vaccines may have on immune mechanisms.[1]

DR. MUNOZ: Along that line, I think I should point out that the time at which pertussigen or these active fractions from *B. pertussis* are given is also important with respect to whether the antibody response is stimulated or suppressed.

DR. ASKELÖF: Dr. Ortez, I am particularly interested in the histamine receptors, and you say that you can block the accumulation of cyclic AMP with the H1 antagonist. Now, H1 works through cyclic GMP. Could you comment on that?

Furthermore, I would like a comment on what concentrations you used in mg/kg body weight.

DR. ORTEZ: The last question first. I believe an effective dose of blocker is about 10 mg/kg.

DR. ASKELÖF: If you are not using well below 1 mg/kg or something like that, you cannot expect it to be specific.

[1] R. Speirs. Suppression and Augmentation of Immune Response to Type III Polysaccharide Depending on Route of Administration of Pertussis Vaccine. Federation Proceedings, 37:1681, 1978.

Could you comment on the H1 receptor too?

DR. ORTEZ: I am not sure I understand the question.

DR. ASKELÖF: You used an H1 antagonist to block the accumulation of cyclic AMP. H1 works through cyclic GMP.

DR. ORTEZ: I have not measured cyclic GMP. There is a report that cyclic GMP is stimulated in pertussis-vaccinated animals with histamine challenge, but that has never been studied with, in effect, an attempt to block it as well.

What is the system where H1 receptors work through cyclic GMP?

DR. ASKELÖF: Wherever you find them.

DR. ORTEZ: I can't comment on that.

DR. GOTSCHLICH: This is merely a technical detail, but it intrigues me. How can you stain with fluorescent
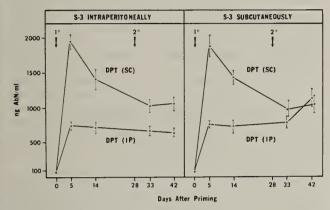
Figure 2. Effect of DTP on antibody titers to S3 (1° 0.5 µg S–3 i.p. or s.c.; 2° 0.5 µg S–3 i.p.): comparison of injection site.

antibody-soluble, cyclic AMP, since I would expect that to be soluble after reaction?

DR. ORTEZ: That is a very legitimate question. People working in this field, those who developed the immuno-fluorescent procedure and others of us working in it, generally agree that we are not looking at a soluble fraction of the cyclic nucleotide, but are looking at a fraction of the cyclic nucleotide that is bound to some protein associated with the cell, perhaps the protein kinase, perhaps the adenylate cyclase, perhaps even a phosphodiesterase. So there is no assurance with this method. In fact, there is probably a pretty good assurance that you are not seeing soluble cyclic nucleotide.

I might mention, however, that when one looks at the relationship between soluble cyclic nucleotide and protein-bound cyclic nucleotide, it has now been demonstrated in the heart, for example, that there is a significant overshoot of soluble cyclic nucleotide production in terms of the ability to saturate the protein kinase. And if you assume that the cyclic AMP in this case is acting through the protein kinase, what you find is perhaps a tenfold increase in total cyclic AMP, but with only a onefold increase, or a doubling, one could saturate all the protein kinase that is there. This suggests that the excess cyclic AMP may not really be as necessary or functional in comparison with that which is bound.

DR. FUKUI: Dr. Chaby, does your lipid X or lipid A give you a positive limulus test?

DR. CHABY: We haven't tried the limulus test yet on the lipid A or the lipid X, but the starting lipopolysaccharide reacts fairly well in the limulus test.

DR. NOVOTNY: In the thiobarbituric acid assay the KDO chromogen is characterized by a maximum at about 550 nanometers. From pertussis-derived membranes we isolated two sugars, one that we have called pertussose,
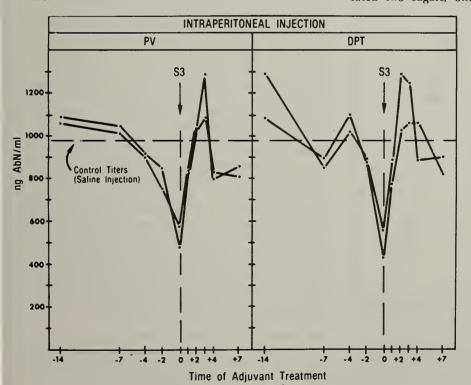
Figure 3. Effect of adjuvant treatment on S3 antibody titers: 5-day response. Pooled data, 5 mice/group.

which is probably a trideoxysugar, and another that is a deoxysugar acid. They could be separated, according to Elbein and Heath, on Dowex-1-carbonate (Fig. 4).

The sugar acid is a kind of pseudo-KDO. In none of the strains we have tested were we able to obtain a maximum in the thiobarbituric acid assay at 550 nm. All of our pertussis "KDOs" had a maximum in the region of 530 nm.

Because of the discrepancy between our results and yours, and because in none of your papers have you stated the maximum of the chromogen, could you say something about this?

DR. CHABY: The maximum of absorbency of the KDO is at 550 nanometers, and we always found this result, of course. We identified KDO by a number of other methods, such as gas liquid chromatography, mass spectrometry of its derivatives, chromatography on thin layer, and so on. There is no doubt about its identity.

DR. BERRY: I would like to comment on the fact that Lipid A from *B. pertussis* is nontoxic. I am sure you are aware that Luderitz has extracted lipid A from several Gram-negative organisms, and he also has found that they are nontoxic. He has characterized them chemically, and it is quite important for this to be done, because some of the biological effects of Lipid A can perhaps then be explained in terms of their chemical structure.
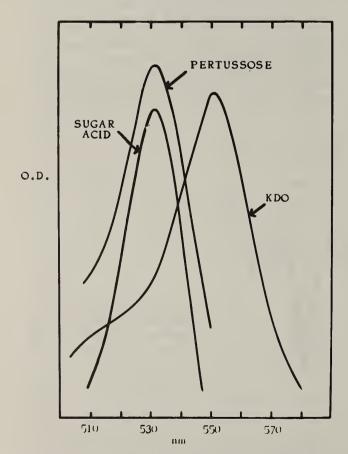
Figure 4. Absorption spectra of thiobarbituric acid assay. Chromogens of *B. pertussis* deoxysugars: "pertussose" and a sugar acid compared with synthetic KDO.

I am sure you have this as one of your objectives, but it will be quite important for you to follow through on this.

I would also like to comment that many of the biological effects of endotoxin or lipopolysaccharide are mediated effects. They are not direct effects. They depend upon factors that are released from macrophages and lymphocytes. In listening to this morning's discussion I have the impression that many of the effects that are being looked at here are perhaps mediated effects and not direct effects of these factors. This can be studied as everyone, I am sure, is aware, by the judicious selection of some inbred strains of animals. I urge the investigators to keep this in mind.

DR. KONG: Have you ever tested the biological effects of your endotoxin preparation on C3H/HeJ mice?

DR. BERRY: May I ask Dr. Munoz a question about the role of the adrenal cortex? It seems to me no one has mentioned this in our discussions. The adrenal medulla has been emphasized, but the adrenal cortex plays a very important role in responses to endotoxin. I wonder if any one has looked at the cortex with respect to pertussigen and some of the other factors. Have you?

DR. MUNOZ: When we first began our investigation on the role of the adrenal gland, the first thing we looked at was, of course, adrenal corticosteroids. We found that 4 mg doses of insoluble hydrocortisone or cortisone protected mice from histamine challenge. But these doses were completely unphysiological.

Soluble preparations didn't work. From this we concluded that the protection obtained with insoluble preparations was not specific. I do not want to say that the cortex does not have anything to do with all the phenomena under investigation.

DR. BERRY: Yes. The shock phenomenon, for example, following endotoxin is due in part at least, if not largely, to the fact that the functioning of the adrenal cortex is blocked by one of the mediators, and these animals behave as if they were adrenalectomized. Our focus has been on the cortex, rather than on the medulla, and I am sure the medulla contributes. But you can use chemicals that block the synthesis of the glucocorticoids and you get the same effect that you do with mediators that have been elicted from macrophages by endotoxin.

DR. MUNOZ: The reason why we have emphasized the medulla, of course, is that Dr. Bergman demedullated mice, and these mice had perfectly healthy cortexes, but they became highly sensitive to histamine.

DR. WOLFF: There is a cell line of adrenal cortex cells called Y1 which responds admirably with steroidogenesis to low concentrations of endotoxins from *E. coli, Salmonella,* and *Serratia,* although I have not tried *B. pertussis.* They also respond to lipid A from *E. coli.* So if you want to check for effects this is an easy assay, easier than the limulus test. The Y1 cell is easy to grow. The system may not work for more specific toxins from *B. pertussis* (Wolff, J. and Cook, G. H., Biochim. Biophys. Acta 413:291–297; 1975).

DR. MUNOZ: I don't have any comment on that, except that pertussigen is not an endotoxin, of course. It is a completely different substance. It would be very nice to check that system with pertussigen.

Part 4. CONTROL TESTING OF PERTUSSIS VACCINE

Chairman: *Frank T. Perkins*
Rapporteur: *Zoltan Csizer*

Opening Remarks

F. T. Perkins

So far in this symposium we have considered the disease, the difficulties in diagnosis, and the growth of the organism. The last session was concerned with the biologically active components of *Bordetella pertussis*. This is where we come to grips with the most critical issues in the immunization against whooping cough. Expressed in its simplest terms, if we knew which antigen of the many contained in the *Bordetella pertussis* was responsible for protection, many of us would not find a symposium such as this of any interest. We would leave the technical problem of the extraction of the protective antigen to the manufacturers and consider that the future of pertussis is solved. How far are we from this situation today? This is the third symposium on pertussis for the International Association of Biological Standardization in almost 20 years, and without wishing to be unkind to those involved in the work, I must say that not a great deal has been achieved. It is true that more and more is known about less and less, but today we are no nearer identifying the protective antigen against whooping cough.

The last 5 years, however, have seen greater interest in the problem, and I do not feel that I am overly optimistic in believing that in the next 10 years the critical questions will be answered. What are the problems?

We have a vaccine that is known to protect against whooping cough, but because whole bacteria are used it may give rise to some local reactions. And unless it has been grown with great care by a process known to yield potent vaccine, the immunizing antigen may not be present in adequate concentration. *Bordetella pertussis* is a fastidious organism demanding special conditions for its optimal growth, and these must be satisfied to produce good vaccine.

The questions of quality control, therefore, are easily asked, but not easily resolved. They are:

1. How do we detect the toxin responsible for the local and generalized reactions? In this context we cannot consider the very rare and serious complications that have been reported.
2. How do we measure the immunizing antigen quantitatively?

Today we can apply several tests for toxicity, but none has been shown to be correlated with local reactions in infants. We subject all pertussis vaccines to the mouse protection test without knowing what antigen is involved in this somewhat complex active challenge test. The test, although having served us well for the last 20 years, may eventually be shown to be inappropriate when the active principle in immunity has been identified.

I have expressed a note of optimism for the future because there is now real evidence that current research is moving closer toward the eventual solution. More research organizations are prepared to support investigations leading toward a common goal; and the World Health Organization, because of its commitments in the developing world, is supporting research into a more stable and less toxic pertussis vaccine.

Today we will hear some of the problems in quality control that we hope to solve in the future.

Pertussis Vaccine: Control Testing Problems

J. Cameron

ABSTRACT

Seven topics are reviewed in this position paper: 1) characterization and maintenance of strains; 2) growth media, detoxification and killing; 3) potency assay; 4) toxicity-testing; 5) opacity; 6) effect of adjuvant; and 7) reference preparations.

Vaccine manufacturers need more guidance in the choice of desirable properties in strains of *Bordetella pertussis* for vaccine manufacture. Improved methods of freeze-drying and seed propagation are also needed to maintain these properties.

The minimal growth requirements of *B. pertussis* are simple, but little seems to have been done to determine the specific metabolic requirements for the production of protective antigen and other biologically active metabolic products such as histamine-sensitizing factor and lymphocytosis-promoting factor. For detoxification, heating cultures or harvests are recommended, whether or not the bacteria are subsequently treated with other reagents, e.g., formalin or thimerosal. Until a new mouse strain that yields more reproducible results in potency testing is more widely distributed, it might be helpful to establish a correlation factor for other strains of mice to make them comparable with those of the control authority.

Because of numerous difficulties with the mouse toxicity test, its most useful role is in lot-to-lot control for individual manufacturers, rather than in trying to detect toxic lots of vaccine when only a minute number of doses in any one lot is implicated. More attention should be paid to the part played by the infant, since the reaction might be a transient sensitization caused by bacterial lipopolysaccharide.

The problems raised by the continued use of two opacity standards, those of the WHO and the Bureau of Biologics, should be resolved soon.

The question is posed as to whether the introduction of adjuvant into vaccines enhances protection in children. The main evidence—increased agglutinin titers—is indirect. The protection enhancing role of adjuvant should be established. It is likely, however, that adjuvant does help reduce reactions by localizing vaccine at the site of injection.

In addition to developing a reference vaccine for use in the potency assay, one or more additional reference preparations should be stored for use in pharmacological studies to ensure better correlation of data between laboratories.

Introduction

My presentation was planned some time ago, and I had intended to cover a wide range of topics. Several of them, however, have already been or will be extensively commented on during this symposium, so further comment would be superfluous. The scope of my report will therefore be limited to aspects of control testing and biological activities of *Bordetella pertussis* that will not be reviewed by others or that seem to merit additional comment. The topics I shall discuss are: characterization and maintenance of strains; growth media, detoxification and killing; potency assay; toxicity testing; opacity; effect of adjuvant; and reference preparations.

Characterization and Maintenance of Strains

In an earlier publication on the problems of control-testing of pertussis vaccine (1) attention was drawn to the extensive data that guided the selection of strains of *Vibria cholerae* for the production of cholera vaccine (2) and the relatively scant data on strains of *B. pertussis* (3). For cholera this wealth of data has not ensured the production of a wholly effective vaccine, whereas effective pertussis vaccines can be produced. Without concluding that a lack of information necessarily leads to the production of effective vaccines, the information given should be less a definitive description of the organism and more a description of the specific properties needed in the organism to ensure the production of

an effective vaccine. It is really not much help to require, as has been done, that the "strain or strains used in the preparation of pertussis vaccine exhibit characteristic colonial and cellular morphology" (3). Accredited strains are readily available for the production of several vaccines—cholera, typhoid, diphtheria and tetanus toxoids, anthrax, brucellosis—yet those for anthrax, brucellosis and diphtheria, and tetanus toxoids, can hardly be said to exhibit "characteristic colonial and cellular morphology." In the case of pertussis vaccine it would be most helpful, particularly in developing countries, if accredited strains were also made available and their location better publicized.

A point not generally appreciated is the difference in performance, even with carefully selected strains, when they are grown on or in different media. For the same strain, potencies between 50 and 60 protective units per single human dose have been reported in cultures grown on Bordet-Gengou medium (4,5), between 30 and 40 units in two-phase, modified Cohen and Wheeler medium (6,7) and no more than 10 units—and then inconsistently—in liquid Cohen and Wheeler medium. Strains for production in fermentors should therefore be chosen on the basis of their performance in shake flasks or fermentors rather than on solid media. This, in effect, is another way of admitting how little we understand the metabolism of B. pertussis in spite of being able to grow it in a chemically defined medium. Since there is no claim, to my knowledge, to a vaccine containing $8-10 \times 10^9$ organisms per single dose, compared with the maximum permissible, $16-20 \times 10^9$ organisms, it can be concluded that it is not yet possible to grow B. pertussis in a way that consistently produces 8–10 protective units per $16-20 \times 10^9$ organisms, a modest doubling of potency. Compare this with the increase in growth and Lf content when the production of diphtheria toxin is transferred from static to fermentor culture.

The most important requirement is for strains to be able to produce at least 4 protective units per dose of vaccine. It is disappointing to assay a vaccine and find that it fails the mouse toxicity test or that it contains too much histamine-sensitizing factor, lymphocytosis-promoting factor, or some other undesirable entity. Thus, apart from the ability to produce protective antigen, strains should also be defined in terms of the properties to be controlled in the vaccine. Unless this is done before vaccine is produced, testing the vaccine

subsequently is like trying to solve a simple linear equation for several unknowns. Mathematically it cannot be done, but in the case of pertussis we keep trying. It has already been shown that the production of protective antigen, histamine-sensitizing factor, and mouse toxic factor does not occur in a fixed ratio within the cell (5). An analysis of strains on this basis can yield strains able to produce much protective antigen and less of the others and offers one means of choosing strains for vaccine production. Potency can be much increased and less desirable properties maintained at acceptable levels, or potency can be maintained at 4 protective units and the content of other entities much reduced.

Since whole-cell vaccines are likely to be preferred in the foreseeable future, and since the development of suitable production strains is laborious, it seems unnecessary to suggest that strains be maintained with care. Yet consider: it is not unusual for strains to be freeze-dried from glucose broth, serum broth, or skimmed milk, to be recovered in a casein hydrolysate solution, passaged a number of times on Bordet-Gengou medium, then given a number of passages in a liquid medium not necessarily of the same composition as the final growth medium. Eventually, possibly somewhat bemused by all these changes of diet, the organism is inoculated into the final growth medium. Surely if strains have been carefully selected they merit better treatment than this.

The possibility of freeze-drying strains from a menstruum as near in composition as possible to that of the final growth medium should be examined. An additional property of the menstruum should be its ability to ensure 80–100% survival on freeze-drying, thus avoiding the selection pressure known to arise in less suitable menstrua. Such menstrua have been developed by the manufacturers of strain 19, Brucella abortus vaccine, and live, attenuated salmonella vaccines that retain almost complete viability for 2 to 3 years. The development of such menstrua for B. pertussis and the subsequent recovery and passage of strains in the final growth medium will at least introduce a consistency sadly lacking in present approaches.

Growth Media, Detoxification, and Killing

There have been only three major developments in growth media in the last 72 years: the introduction of Bordet-Gengou medium in 1906, the medium on which B. pertussis was first isolated (4);

the introduction of Hornibrook's casein hydrolysate medium in 1939, when blood and serum were eliminated for the first time (8); and the introduction of Hornibrook's chemically defined medium in 1940 (9). All media in use today are essentially based on one of these.

The basic growth requirements are one amino acid (L-glutamic acid or proline), a source of sulfur (L-cysteine, L-cystine, or glutathione), nicotinic acid or nicotinamide, and salts. The optimum salt requirements are not known. It would seem, starting from such a simple, well-defined baseline, that the next steps would be to define specific conditions for the production of protective antigen and other entities that may form the basis of a particular study. The first priority should be controlled production of protective antigen. Such studies could also reveal the extent to which the production of histamine-sensitizing factor and lymphocytosis-promoting factor, for example, are independently controlled or if they are produced in different but constant ratios in different strains. We also need further studies along the lines pioneered by Demain (10).

Methods of detoxification and killing are well established: heat (56° for 30 minutes) or heat followed by the addition of formalin; formalin alone; or thimerosal alone. Heat rapidly inactivates the heat-labile or dermonecrotic toxin without damaging protective antigen and should be used either by itself or with subsequent addition of formalin or thimerosal. It is also advisable, if heat is used, to introduce the other reagent as soon as possible afterward to reduce the risk of contamination.

Potency Assay

There is little doubt that of the many biological products for which there are established potency assays, pertussis is the most troublesome. Manclark reported that 15–20% of lots regarded as of acceptable potency by manufacturers failed confirmatory assay by the Bureau of Biologics (11). This may seem unusual, considering that reference vaccines have been in use for many years. One of the problems, however, has been lack of consistency in response in the mouse-reference vaccine-challenge system. Inconsistency of response to the reference vaccine is not general, but where it does occur it is extremely troublesome, for it can result in the rejection of assays involving hundreds of mice. On the other hand, if the failure to respond concerned

a single sample of vaccine in the assay, only the results for the sample would be rejected and much useful data could still be acquired from the test.

A collaborative assay organized by the WHO to determine the role played by the challenge found that a uniform method for the preparation of the challenge reduced but did not eliminate heterogeneity of results (12). In other experiments where a freeze-dried reference vaccine was used and where the challenge was recovered from liquid nitrogen no great improvement was noted (Cameron, unpublished). My own conclusion at the time, wrong though it proved to be, was: "There is an inherent unsoundness in the assay, perhaps in the use of the intracerebral challenge, but clearly much investigation is needed to validate such an assertion" (1). Current work by Manclark and his colleagues (personal communication) seems to make it clear that the mouse itself is the major variable in the system.

What follows from the fact that different ImD_{50}s are recorded in different strains of mice for the same reference vaccine? One question, important from the control point of view, is whether different mice will give the same estimate of potency on the same sample of vaccine if they are already known to give different ImD_{50} values for the reference vaccine. This is less important where a manufacturer is responsible for his own product, but in the United States, where samples have to be submitted to the Bureau of Biologics for confirmatory testing, it may explain the high failure rate reported by Manclark (11).

This may not be a general problem; some laboratories are known to record the same ImD_{50} value for the same reference preparation and for the same samples of vaccine, so presumably they are using genetically similar mice. Until such mice are more widely distributed, however, it would be a useful precaution to determine whether a correction factor should be applied to one's own mice to make them comparable to those used by the control authority. This would still be only a small step in the right direction and might do little to compensate for the wide variability in assay results reported by Finney, Sheffield, and Holt (13). In 1970 these authors analyzed the data from 32 consecutive assays involving groups of 32 mice and a range of three fivefold dilutions and found an eightfold range in ImD_{50} values. Of the 32 assays, 13 even fell outside the 95% confidence limits. Similar treatment of data for 1971, 1972, and 1973 con-

firmed the findings. The authors' aim was to determine if data on a series of assays could be pooled to provide a single definitive dose-response curve for the reference vaccine that could be used for all subsequent assays without further observations on the reference. They concluded that this was inadvisable and that it was desirable to retain "the usual statistical practice: relative potency should be estimated from simultaneous trials of the reference and test vaccines, in order to eliminate variations in responsiveness of subjects from one occasion to another." Examination of similar data of my own, involving a different strain of mouse, essentially confirmed these findings. The responsiveness of this particular mouse strain became so poor at one time that it could no longer be used for assays.

It is trite to say that an alternative method of measuring the potency of pertussis vaccine would be invaluable. But regardless of which particular line of investigation is undertaken in an attempt to provide a more effective or safer vaccine, all new preparations ultimately have to be assayed in mice. A single test involving five samples and a reference vaccine, with appropriate controls on the challenge, needs 350 to 400 mice of one sex and similar age, within a weight range of 4 grams. The test lasts 4 weeks (2 for immunization and 2 for observation after challenge). If 10 samples are tested per week, not an unusual number, it means that about 3,000 mice are permanently on test. This is fairly representative of routine testing for a vaccine containing three different strains of *B. pertussis*. Add to this the samples that may be generated in a research program and it is understandable why an alternative form of assay would be welcome. It is also quite possible that the cost of mice and the difficulties in housing them may have been partly responsible for research programs moving in directions that avoid the necessity for large scale potency testing. While this may be understandable, it is still really only postponing the inevitable. Until an alternative test system is evolved, a meaningful program of research on the two properties of pertussis vaccine that come to mind most readily—efficacy and safety—will continue to be expensive both in technical staff and the provision of mice.

Toxicity Testing

There is always some concern when pertussis vaccine is injected into infants, not so much because its potential reactivity has been well docu-

mented, but because it has almost certainly been greatly exaggerated. Any fruitful discussion on the toxicity testing of pertussis vaccine must presumably start from a basis of established fact. That seems to be the first and major problem— a lack of factual data. Europe and North America may be able to afford the luxury of debating the proposition that the cure is worse than the disease, but in developing countries, where mortality may be as high as 25% (D.J.M. Tarantola, personal communication), it is our responsibility to ensure that these two features of the vaccine are seen in proper perspective and that concern about reactivity is not elevated to a level where it can prejudice an immunization campaign.

What are we attempting to control when we do a mouse toxicity test? Clearly we are trying to decide whether or not a particular lot of vaccine is suitable for release. On the other hand, presumably every lot of vaccine released in the last 5 to 10 years has been safe by this standard, yet reactions are still observed in infants.

The report by Pittman and Cox (14) reviewed the development of the toxicity test in the United States up to 1965. Even in this report, however, there were indications of the type of problem other workers would encounter later. The 7-day weight gain of saline-injected control mice increased from 4 grams in 1955 to 6 grams in 1963. The injection of adsorbed vaccines caused greater initial weight loss as a result of the presence of adjuvant. Later it was reported that the weight gain of some strains of mice was quite unaffected by the injection of vaccine (1,15,16). A potentially dangerous interpretation put on this observation was that a particular vaccine was absolutely safe because it did not cause weight loss in mice! Later it was shown that the weight gain of mice is affected by the temperature at which they are held, the amount of light to which they are exposed, and the size of their cage (17).

The dilemma posed by these observations is whether to keep trying to refine the test or to look for alternative tests. There is no doubt that the test can detect toxic lots of vaccine; in a valuable paper, Cohen, van Ramshorst, and Drion (18) correlated depressed weight gain in mice with raised rectal temperatures and reactions (not described) in infants. It should be emphasized that all lots of vaccine examined in this study had passed the mouse toxicity test, but the reactive ones caused a significantly lower weight gain than the nonreactive ones.

These observations established what is probably the most useful role for the test, namely providing lot-to-lot control for an individual manufacturer. The vaccine in the study was DTP-polio and the "reactive" lots contained a pertussis component that had been separated from the whole culture by precipitation with acid rather than by centrifugation. The toxic factor, not identified, was heat-stable and was presumably irreversibly adsorbed onto the bacteria on precipitation. It could have been lipopolysaccharide, since growth of Gram-negative organisms in fermentors, depending on the rate of growth, may be accompanied by the release of large quantities of lipopolysaccharide into the growth medium (19). Such cell-free lipopolysaccharide is much more reactive than when it occurs as an integral component of the cell wall (Cameron, unpublished).

The dilemma in trying to develop a more meaningful toxicity test (more accurately, a reactivity test) for pertussis vaccines is the sporadic manner in which reactions occur. Even when a reaction has been observed it is still likely that thousands if not hundreds of thousands of doses of vaccine of the same lot have been injected without incident. This suggests that the infant, too, plays a part in the reaction, probably through some form of hypersensitivity. There is no clear evidence for such a reaction, but perhaps some speculation is permitted.

There are at least two analogous situations in veterinary medicine. First, the injection of non-adsorbed hemorrhagic septicemia (*Pasteurella multocida*) vaccine into buffalo calves can occasionally cause a dramatic collapse, as can the injection into beef calves of a combined *Escherichia coli/Salmonella dublin* vaccine for the prevention of enteritis. In both cases, the syndrome is almost completely abolished if the vaccines are adsorbed. Second, the intravenous injection into calves of as little as 0.2 ml. of the supernatant of a fermenter-grown culture of *E. coli* can cause collapse within 5 minutes followed by recovery in 5 hours (20,21).

If these are hypersensitivity reactions, the first question to be answered is, "How does sensitization occur?" It seems unlikely that what might be called "homologous" sensitization is involved, particularly in the case of *B. pertussis*, i.e., prior sensitization to *B. pertussis* itself. The study by Malkiel and Hargis (22) suggests that heterologous sensitization might well be possible. These authors found that when mice were sensitized with *B. pertussis*, a fatal reaction could be induced in more than half of them when they were challenged later with lipopolysaccharides from *B. pertussis*, *B. bronchiseptica*, *B. abortus*, *Serratia marcescens*, *S. typhi*, or *E. coli*.

If heterologous sensitization is then possible, how does it occur? In infancy, when the intestinal mucosa is not wholly impermeable, an intestinal upset accompanied by proliferation of Gram-negative bacteria will also be accompanied by the release of some lipopolysaccharide into the lumen of the intestine, in a manner possibly analogous to the release of lipopolysaccharide into the growth medium when Gram-negative organisms are grown in a fermentor (19). Enough to induce sensitization may then be absorbed.

There is further evidence to support these suggestions. Hannik (23), who has made a major study of reactions in infants, noted that of 16 reported reactions, 11 occurred after the first injection of vaccine, 4 after the second, and 1 after the third. For homologous sensitization the reverse might have been expected. Sensitization to lipopolysaccharide is transient (24) and, for a shock reaction to occur, injection of vaccine would have to coincide with the hypersensitive state. This might also explain the infrequency of reactions. In the case of the *E. coli/S. dublin* vaccine used in calves a situation is known (Cameron, unpublished) in which vaccination of a group of calves was stopped because several animals collapsed on injection. When vaccination was resumed with the same lot of vaccine a few days later there were no further reactions.

If indeed reactions occur more as a result of hypersensitivity in an infant than from the injection of a particularly reactive lot of vaccine, can anything be done to prevent them? The sensitizing agents must first be indentified and the reactions they induce must be better understood. The serological specificity of lipopolysaccharides, based on electrophoretic studies using antisera produced by a series of injections into rabbits, tends to obscure the fact that these same molecules can produce a transient sensitization and even a short-lived protection against challenge with heterologous lipopolysaccharides. One precaution might be to base decisions to inject not so much on an infant's health on the day of injection as over the preceding 7 days, certainly the previous 2. Work could also be directed toward developing a model for sensitization so that vaccine lots could be screened for this property. Such a test might never detect a potentially toxic or reactive lot of vaccine, but if

the type of reaction seen in infants is caused by hypersensitivity, it seems reasonable to examine vaccines for their sensitizing ability. Additional emphasis could be placed on thorough washing of the bacteria in the vaccine, bearing in mind the effect of acid-precipitation, for example, and on minimizing the potential for lysis in the vaccine as it ages.

Aspirin might also help prevent reactions. Occasionally, parents have been advised to give their infant aspirin after vaccination. Although I understand this should not be done, there are experimental data to show that aspirin prevents anaphylactic shock in rabbits (25,26). It might be useful to bear this in mind in studies on sensitization to pertussis vaccine.

Opacity

The purpose of a reference opacity preparation is to provide a simple, visual means of ensuring lot-to-lot reproducibility of the density of a suspension of *B. pertussis*. This in turn reasonably assures that each dose of vaccine contains the same number of bacteria. But this method of comparison does not take into account possible inherent differences in the size or shape of cells among various strains or the variations that may arise from different conditions of growth. Whole cell counts are more accurate. Other parameters suggested are DNA, nitrogen content, and dry weight, but it has not been shown that any of these measurements yield better information on which to base judgments. In recent years, however, it was noted that the reference preparation used in the United States is almost twice as dense as that of the WHO, although both are normally equivalent to a suspension of *B. pertussis* containing 10×10^9 bacteria per ml (10 opacity units).

This raises several questions. Since potency and toxicity are assayed on dilutions of the vaccine itself, does this mean that vaccines adjusted to the WHO standard are twice as potent and half as toxic, organism for organism, as those made to the U.S. standard? A new opacity standard (27) has been recommended to the WHO for adoption and is already in use in many laboratories. This standard differs from the previous WHO standard in the ratio of 1.1:1. An immediate practical advantage to U.S. manufacturers in adopting it would be almost to double production, quite literally by a stroke of the pen! Of more concern will be the question of potency, since the present minimum

requirement of 4 protective units per single dose with a lower value of 2.8 units in a single assay will have to be raised to almost 8 units with a lower value of 5.6 units. Another advantage will be the additional safety factor introduced by halving the number of bacteria per dose.

If, indeed, U.S. vaccines contain twice as many bacteria per dose as European and British vaccines, one might expect U.S. vaccines to be twice as reactive as British vaccines. Is this the case? It has been shown that as far as British vaccines are concerned, a doubling of the dose in the toxicity test, which brings the preparation to the same opacity as U.S. vaccines, yields a preparation that barely passes the test (15). U.S. vaccines usually pass the test comfortably.

Effect of Adjuvant

The role of adjuvant in the host response to pertussis vaccine is far from clear. In the mouse-protection test, adsorbed vaccines are believed to perform better than nonadsorbed vaccines, and aluminum phosphate as adjuvant, in turn, is thought to be marginally better than aluminum hydroxide (L. Higy Mandic, J. D. van Ramshorst, personal communications). On the other hand, there is some evidence that aluminum hydroxide actually depresses the protective response in mice (7, 28). Bearing in mind the now well documented vagaries of the mouse-protection test (13) it seems likely that adjuvant exerts minimal, if any, effect on potency.

Certainly it would require a sustained series of assays with a small number of vaccines, something yet to be done, to validate the claims made for adjuvant. This view is supported to some extent by the fact that adsorbed and nonadsorbed vaccines are regularly included in the same assay and by the fact that most if not all reference preparations are nonadsorbed, whereas the great majority of vaccines are adsorbed. If there is an enhanced response it is not great enough to make the slopes of the dose-response curves nonparallel. In contrast, the effect of adjuvant on diphtheria and tetanus toxoids is so great that plain and adsorbed toxoids cannot be compared in the same assay.

Two explanations have been offered for the inability to demonstrate a clear effect of adjuvant: that pertussis is a whole-cell vaccine or alternatively that it is a Gram-negative whole-cell vaccine. As generalizations, neither is acceptable. Recently bet-

ter and longer lasting protection was reported in man with adsorbed cholera vaccine (29), and in the veterinary field, the immunogenicity of hemorrhagic septicemia *(P. multocida)* vaccine is strikingly increased in the presence of adjuvant. Both of these are Gram-negative vaccines. In the case of experimental *S. typhimurium* vaccine, which can be shown to prevent septicemia in mice, no effect of adjuvant can be shown. In this model, however, as with pertussis, the agglutinin response is strikingly enhanced. This should suggest caution in interpreting the increase in agglutinin response after the injection of adsorbed pertussis vaccine into infants as being anything but an indication of exposure to *B. pertussis* (30). If protection in mice parallels protection in children, as was deduced from the MRC trials in the United Kingdom (31), and if adjuvant cannot be shown to enhance protection in mice, the logical conclusion must be that it does not enhance protection in infants either. It seems advisable to clarify this point before the presumed effect of adjuvant on antibody response becomes any more deeply enshrined in the folklore of pertussis.

One property of adjuvant that may well help reduce vaccination reactions is its ability to localize the vaccine at the site of injection. The introduction of adjuvant into the two veterinary vaccines mentioned above almost completely abolishes the collapse syndrome occasionally induced by the nonadsorbed vaccines.

Reference Preparations

Work on pertussis vaccine has tended to suffer from the assumption that pertussis vaccine is almost as well defined as sodium chloride. Strains and vaccines differ serologically, different growth media are used, different methods of killing are used. The assay methods are almost certainly not subtle enough to detect what may be major differences between preparations. For this reason it would be highly beneficial if large stocks of freeze-dried reference preparations could be prepared for use in developing new lines of investigation. These preparations would be used, as are the corresponding preparations for the potency assay, in establishing substandards. Experience with freeze-dried reference preparations shows that they can be expected to have a life of at least 5 years. Use of substandards in turn should enable firm data bases to be developed for laboratory-to-laboratory comparisons. Such reference preparations will be especially needed if

the suggestion that reactions may be due to hypersensitivity proves fruitful.

REFERENCES

1. Cameron, J. Problems associated with the control testing of pertussis vaccine. Adv. Appl. Microbiol. 20:57–80; 1976.
2. Requirements for cholera vaccine. WHO Tech. Rep. Ser. 413; 1969.
3. Requirements for pertussis vaccine. WHO Tech. Rep. Ser. 274; 1964.
4. Bordet, J.; Gengou, O. Le microbe de la coqueluche. Ann. Inst. Past. 20:731–741; 1906.
5. Cameron, J. Variation in *Bordetella pertussis*. J. Pathol. Bacteriol. 94:367–374; 1967.
6. Cohen, S. M.; Wheeler, M. W. Pertussis vaccine prepared with phase I cultures grown in fluid medium. Am. J. Public Health. 36:371–376; 1946.
7. Cameron, J.; Knight, P. A. Interaction of components of triple antigen (diphtheria, tetanus, pertussis: DPT). Proceedings of the Symposium on Combined Vaccines. Yugoslavian Academy of Science and Arts, Zagreb: 1972:55–67.
8. Hornibrook, J. W. Cultivation of phase I *H. pertussis* in a semi-synthetic liquid medium. Public Health Rep. 54:1847–1851; 1939.
9. Hornibrook, J. W. Nicotinic acid as a growth factor for *B. pertussis*. Proc. Soc. Exp. Biol. Med. 45:598–599; 1940.
10. Demain, A. L. The role of culture collections in the era of molecular biology. Washington, D.C.: American Society for Microbiology; 1976:56–61.
11. Manclark, C. R. The current status of pertussis vaccine: an overview. Adv. Appl. Microbiol. 20:1–7; 1976.
12. Murata, R.; Perkins, F. T.; Pittman, M.; Scheibel, I.; Sladky, K. International collaborative studies on the pertussis vaccine potency assay: part played by the challenge in the mouse-protection test. Bull. WHO 44:673–687; 1971.
13. Finney, D. J.; Holt, L. B.; Sheffield, F. Repeated estimations of an immunological response curve. J. Biol. Stand. 3:1–10; 1975.
14. Pittman, M.; Cox. C. B. Pertussis vaccine testing for freedom-from-toxicity. Appl. Microbiol. 13:447–456; 1965.
15. Cameron, J. Pertussis vaccine: mouse-weight-gain (toxicity) test. Dev. Biol. Stand. 34:213–215; 1976.
16. van Ramshorst, J. D. Toxicity control of pertussis vaccines by the mouse-weight-gain test. Prog. Immunobiol. Stand. 3:324–326; 1969.
17. Cameron, J. The influence of environment on the mouse-weight-gain test for estimating the toxicity of *Bordetella pertussis* vaccines. Prog. Immunobiol. Stand. 3:319–323; 1969.
18. Cohen, H.; van Ramshorst, J. D.; Drion, E. F. Relation between toxicity tests in mice and reactions in children using four lots of quadruple vaccine (DPT-polio). Symp. Series Immunobiol. Stand. 10:53–62; 1969.
19. Crutchley, M. J.; Marsh, D.G.; Cameron, J. Free endotoxin. Nature 214:1052; 1967.
20. Cameron, J. Responses induced in mice by the injection of mouse-virulent strains of *Escherichia coli*. Symp. Series Immunobiol. Stand. 15:65–72; 1971.
21. White, G. H.; Cameron, J. The "collapse syndrome" in young calves. Vet. Rec. 86:328–329; 1971.
22. Malkiel, S.; Hargis, B. J. Anaphylactic reactions in mice induced by *Bordetella pertussis* lipopolysaccharide. J. Allergy 35:306–312; 1964.

23. Hannik, C. A. Major reactions after DPT-Polio vaccination in the Netherlands. Symp. Series Immunobiol. Stand. 13:161–170; 1970.

24. Homma, J. Y. The Shwartzman phenomenon in the mouse. Jpn. J. Exp. Med. 22:17–22; 1952.

25. Campbell, B. Inhibition of anaphylactic shock by acetylsalicylic acid. Science 108:478–479; 1948.

26. Lepper, M. H.; Caldwell, E. R.; Smith, P. K.; Miller, B. F. Effects on anaphylactic shock of salicylates, aminopyrine and other chemically and pharmacologically-related compounds. Proc. Soc. Exp. Biol. Med. 74:254–258; 1950.

27. Perkins, F. T.; Sheffield, F. W.; Outschoorn, A. S.; Hemsley, D. A. An international collaborative study on the measurement of opacity of bacterial suspensions. J. Biol. Stand. 1:1–10; 1973.

28. Novotny, P.; Brookes, J. E. The use of *Bordetella pertussis* preserved in liquid nitrogen as a challenge suspension in the Kendrick mouse protection test. J. Biol. Stand. 3:11–29; 1975.

29. Indian Council of Medical Research. Annual Report of Director-General. New Delhi; 1975:13–14.

30. Preston, N. W.; Mackay, R. I.; Bamford, F. N.; Crofts, J. E.; Burland, W. L. Pertussis agglutinins in vaccinated children: better response with adjuvant. J. Hyg. (Camb.) 73:119–125; 1974.

31. Vaccination against whooping-cough: relation between protection in children and results of laboratory tests. Br. Med. J. 2:454–462; 1956.

Comparative Mouse Toxicity Testing of Diphtheria-Pertussis-Tetanus Vaccines

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ABSTRACT

Thirty different preparations (nonadsorbed and adsorbed diphtheria and tetanus toxoids; diphtheria-pertussis; tetanus-pertussis; and DTP vaccines), and four control vaccines were tested in the mouse toxicity test. Regression analysis of the dose-response functions, analysis of variance, and Gabriel's multiple comparison test were used for the statistical evaluation.

BWD_1, BWD_{5-2}, and BWD_7 data were characteristic for early, late, and total toxicity, respectively. Aluminum phosphate (1.5 mg) had an early weight-decreasing toxicity without lethal effect. Pertussis vaccine (10 IOU) possessed both weight-decreasing and lethal toxicity. The adsorbent, which did not possess late toxicity itself, enhanced the late toxicity of pertussis vaccine. According to the BWD_7 data, the diphtheria toxoids were more toxic in the mouse toxicity test than the tetanus toxoids, and the combined vaccines containing diphtheria toxoid were more toxic than those that contained tetanus toxoid. The adsorbed DTP vaccines were the most toxic combinations.

The additional toxicity of the combined vaccines is due to the toxicity-enhancing effect of the adsorbent and the diphtheria toxoid component.

INTRODUCTION

Since the establishment of the U.S. requirements for pertussis vaccine in 1948, the mouse toxicity test of pertussis vaccine has been extensively discussed (1–11). The characteristics of the different toxic factors of *Bordetella pertussis* and the toxicity of aluminum hydroxide and aluminum phosphate adsorbents are well established (1, 3, 12–13). Our experience over several years, however, shows that the adsorbed diphtheria-pertussis-tetanus (DTP) vaccines have some additional toxicity that cannot be explained solely by the toxicity of the pertussis component or of the adsorbent. Our aim was to find the source(s) of this additional toxicity and to explain the phenomenon.

MATERIALS AND METHODS

Strains of Bacteria

The following strains were used:

Corynebacterium diphtheriae P.W.8; *Clostridium tetani* Harvard; and *Bordetella pertussis* 41405 (serotype 1.2.3), 59 (serotype 1.3), 324E (serotype 1.2), 358E (serotype 1.2), CN2894 (serotype 1.2), CN2896 (serotype 1.2), and CN2897 (serotype 1.2).

Culture Media

C. diphtheriae was cultivated in Linggood medium, *C. tetani* was cultivated in beef heart broth digested with pepsin and trypsin (14), and *B. pertussis* was cultivated in modified Cohen-Wheeler medium (15).

Cultivation

C. diphtheriae and *C. tetani* were grown in static cultures (16); *B. pertussis* was grown in fermentor cultures (17).

Preparation and Purification of Toxoids

Diphtheria and tetanus toxoids were purified by precipitation with trichloroacetic acid (18). The purity of the diphtheria and tetanus toxoids varied from 550 to 700 Lf/mg protein nitrogen and from 800 to 1,000 TCP/mg protein nitrogen respectively. Diphtheria toxoids purified by ammonium sulfate precipitation had a purity of 1,500 Lf/mg protein nitrogen (19).

Preparation of Pertussis Vaccine

Acid-precipitated, merthiolate-treated, and heat-inactivated *B. pertussis* suspensions were used (20). The ratio of serotype 1.2.3; serotype 1.3; and serotype 1.2 in the pertussis vaccines was 3:3:4.

Composition of Vaccines

The composition and dose of vaccines used in the mouse toxicity tests are given in Table 1.

Table 1. Composition and Dose of Vaccines Used in Mouse Toxicity Test

Vaccines (abbreviations)	Composition	Dose		Vaccines (abbreviations)	Composition	Dose	
Control (C)	Isotonic saline	—		Adsorbed tetanus 1 (T1A)	Tetanus toxoid	500	TCP
Adsorbent control (CA)	$AlPO_4$	1.5 mg			$AlPO_4$	1.5 mg	
Pertussis (P)	Pertussis bacteria	10	IOU	Adsorbed tetanus 2 (T2A)	Tetanus toxoid	50	TCP
Adsorbed pertussis (PA)	Pertussis bacteria	7.5	IOU		$AlPO_4$	1.5 mg	
	$AlPO_4$	1.5 mg		Adsorbed tetanus 3 (T3A)	Tetanus toxoid	5	TCP
Diphtheria 1 (D1)	Diphtheria toxoid	1000	Lf		$AlPO_4$	1.5 mg	
Diphtheria 2 (D2)	Diphtheria toxoid	100	Lf	Adsorbed diphtheria-	Diphtheria toxoid	1000	Lf
Diphtheria 3 (D3)	Diphtheria toxoid	10	Lf	pertussis 1 (DP1A)	Pertussis bacteria	7.5	IOU
Tetanus 1 (T1)	Tetanus toxoid	500	TCP		$AlPO_4$	1.5 mg	
Tetanus 2 (T2)	Tetanus toxoid	50	TCP	Adsorbed diphtheria-	Diphtheria toxoid	100	Lf
Tetanus 3 (T3)	Tetanus toxoid	5	TCP	pertussis 2 (DP2A)	Pertussis bacteria	7.5	IOU
Diphtheria-pertussis 1	Diphtheria toxoid	1000	Lf		$AlPO_4$	1.5 mg	
(DP1)	Pertussis bacteria	10	IOU	Adsorbed diphtheria-	Diphtheria toxoid	10	Lf
Diphtheria-pertussis 2	Diphtheria toxoid	100	Lf	pertussis 3 (DP3A)	Pertussis bacteria	7.5	IOU
(DP2)	Pertussis bacteria	10	IOU		$AlPO_4$	1.5 mg	
Diphtheria-pertussis 3	Diphtheria toxoid	10	Lf	Adsorbed tetanus-pertussis	Tetanus toxoid	500	TCP
(DP3)	Pertussis bacteria	10	IOU	1 (TP1A)	Pertussis bacteria	7.5	IOU
Tetanus-pertussis 1 (TP1)	Tetanus toxoid	500	TCP		$AlPO_4$	1.5 mg	
	Pertussis bacteria	10	IOU	Adsorbed tetanus-pertussis	Tetanus toxoid	50	TCP
Tetanus-pertussis 2 (TP2)	Tetanus toxoid	50	TCP	2 (TP2A)	Pertussis bacteria	7.5	IOU
	Pertussis bacteria	10	IOU		$AlPO_4$	1.5 mg	
Tetanus-pertussis 3 (TP3)	Tetanus toxoid	500	TCP	Adsorbed tetanus-pertussis	Tetanus toxoid	5	TCP
	Pertussis bacteria	10	IOU	3 (TP3A)	Pertussis bacteria	7.5	IOU
Diphtheria-tetanus-	Diphtheria toxoid	1000	Lf		$AlPO_4$	1.5 mg	
pertussis 1 (DTP1)	Tetanus toxoid	500	TCP	Adsorbed diphtheria-	Diphtheria toxoid	1000	Lf
	Pertussis bacteria	10	IOU	tetanus-pertussis 1	Tetanus toxoid	500	TCP
Diphtheria-tetanus-	Diphtheria toxoid	100	Lf	(DTP1A)	Pertussis bacteria	7.5	IOU
pertussis 2 (DPT2)	Tetanus toxoid	50	TCP		$AlPO_4$	1.5 mg	
	Pertussis bacteria	10	IOU	Adsorbed diphtheria-	Diphtheria toxoid	100	Lf
Diphtheria-tetanus-	Diphtheria toxoid	10	Lf	tetanus-pertussis 2	Tetanus toxoid	50	TCP
pertussis 3 (DPT3)	Tetanus toxoid	5	TCP	(DTP2A)	Pertussis bacteria	7.5	IOU
	Pertussis bacteria	10	IOU		$AlPO_4$	1.5 mg	
Adsorbed diphtheria 1	Diphtheria toxoid	1000	Lf	Adsorbed diphtheria-	Diphtheria toxoid	10	Lf
(D1A)	$AlPO_4$	1.5 mg		tetanus-pertussis 3	Tetanus toxoid	5	TCP
Adsorbed diphtheria 2	Diphtheria toxoid	100	Lf	(DTP3A)	Pertussis toxoid	7.5	IOU
(D2A)	$AlPO_4$	1.5 mg			$AlPO_4$	1.5 mg	
Adsorbed diphtheria 3	Diphtheria toxoid	10	Lf				
(D3A)	$AlPO_4$	1.5 mg					

Mice

Random bred CFLP mice of both sexes, weighing 15 to 16 g, were used. In each group of 12 mice the mean body weight was 15.5 g. The body weight data of mice within the groups were homogeneous (21).

Toxicity Test

Toxicity tests were carried out according to the WHO requirements for pertussis vaccine (22). Each vaccine was tested in six independent toxicity tests. In all, 72 mice were used for each vaccine. Six male and six female mice of 16 and 15 g respectively were used in each test; thus the total body weight in one group of 12 mice on the day of immunization was 186 g.

Statistical Analysis

The log dose function of the body weight difference values can be regarded as linear. (The dif-ference between the body weight on the day of immunization and that on the ith day after immu-nization is abbreviated as BWD_i.) Regression analysis of the dose response functions, analysis of variance, and Gabriel's multiple comparison test were used for the statistical evaluation (23).

RESULTS

The mean body weight changes of mice after injection with different singular and combined vac-cines are shown in Figures 1–5.

The regression analysis of the data of BWD_1 is given in Table 2. The regression lines are linear, but not parallel. According to Gabriel's multiple comparison test, however, the deviation from the identity of the regression lines is mainly due to the difference of treatment means (Tables 3,4). Signifi-cant difference has been found between the toxicity of plain and adsorbed vaccines. The difference is caused by the early toxicity of aluminum phosphate.

Table 2. Regression Analysis of the BWD$_1$ Data Obtained with Different Vaccines

Vaccine	A + B × T	S.E. of A	S.E. of B	Regr. SSq	D.F.	Lin. F	D.F.	Sign.
D	−1.26 + 0.81 × T	0.34	0.16	3.3605	16	0.5179	1/150	NS
T	0.13 + 0.36 × T	0.34	0.16	1.0282	16	0.9085	1/150	NS
DP	−1.00 + 0.53 × T	0.34	0.16	1.5433	16	0.3181	1/150	NS
TP	−0.14 + 0.18 × T	0.34	0.16	5.8293	16	1.5458	1/150	NS
DTP	−0.12 + 0.24 × T	0.34	0.16	4.0795	16	0.3436	1/150	NS
DA	−1.52 + 0.25 × T	0.34	0.16	8.4847	16	0.0140	1/150	NS
TA	−1.37 + 0.32 × T	0.34	0.16	9.6097	16	1.0869	1/150	NS
DPA	−0.62 − 0.09 × T	0.34	0.16	5.9334	16	0.1449	1/150	NS
TPA	−0.64 − 0.10 × T	0.34	0.16	2.6045	16	0.0093	1/150	NS
DTPA	−0.67 − 0.12 × T	0.34	0.16	3.4570	16	0.1695	1/150	NS

F test for parallelism: 3.53   D.F.: 9/150   P5% = S   F test for identity: 28.14   D.F.: 9/150   P5% = S   Common regression coefficient: 0.24   Its S.E.: 0.05
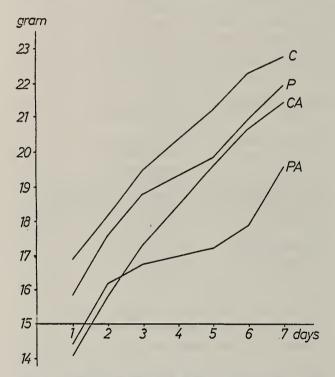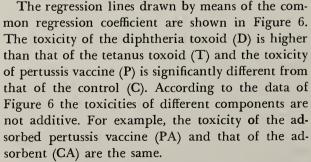

Figure 1. Mean body weight change of 72 mice after inoculation of control vaccines.

Figure 2. Mean body weight change of 72 mice after inoculation of plain toxoids.

The regression lines drawn by means of the common regression coefficient are shown in Figure 6. The toxicity of the diphtheria toxoid (D) is higher than that of the tetanus toxoid (T) and the toxicity of pertussis vaccine (P) is significantly different from that of the control (C). According to the data of Figure 6 the toxicities of different components are not additive. For example, the toxicity of the adsorbed pertussis vaccine (PA) and that of the adsorbent (CA) are the same.

Statistical analysis of the BWD$_2$ data gave similar results (Fig. 7). Tetanus toxoid is significantly less toxic than adsorbed diphtheria toxoid (DA), ad-

sorbed diphtheria-pertussis vaccine (DPA), and adsorbed DTP (DTPA). The toxicity of pertussis vaccine is enhanced by diphtheria toxoid. The vaccines containing D are more toxic than the corresponding vaccines containing T, but the differences are not statistically significant.

The toxic effect of lymphocytosis-promoting factor (LPF) can be characterized by the data of BWD$_3$ and BWD$_{5-2}$ (the difference between the body weight on the 5th and 2nd days after injection of vaccine). The regression lines computed from the BWD$_3$ data are parallel but not identical. The regression line of T is different from all the dose

Table 3. Gabriel's Multiple Comparison Test of Regression Coefficients Computed from the BWD$_1$ Data

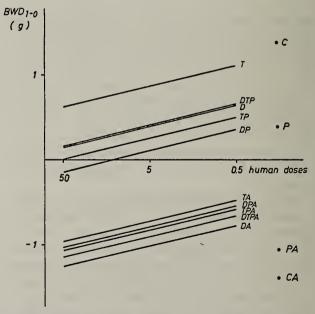| Vaccine	Order	Order, Minimum	Order, Maximum
D	1	1	7
T	3	1	10
DP	2	1	10
TP	7	1	10
DTP	6	1	10
DA	5	1	10
TA	4	1	10
DPA	8	2	10
TPA	9	2	10
DTPA	10	2	10

Table 4. Gabriel's Multiple Comparison Test of Treatment Means Computed from the BWD$_1$ Data

| Vaccine	Order	Order, Minimum	Order, Maximum
D	2	1	5
T	1	1	4
DP	5	2	5
TP	4	1	5
DTP	3	1	5
DA	10	6	10
TA	6	6	10
DPA	7	6	10
TPA	8	6	10
DTPA	9	6	10

tween the loss of body weight and the number of deaths was not found in all tests. The aluminum phosphate gel has a statistically significant early
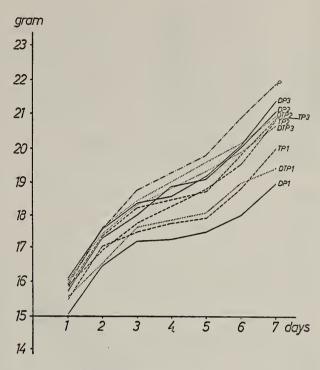
Figure 3. Mean body weight change of 72 mice after inoculation of plain combined vaccines.

response lines except line D. These differences are statistically significant (Fig. 8). The regression analysis of the BWD$_{5-2}$ data is shown in Table 5. The regression lines are parallel, but not identical (Table 6). The toxoids are less toxic than the combined vaccines (Fig. 9). These differences are statistically significant. The control toxoids C and CA have no late toxicity. The toxic effect of *B. pertussis* LPF is greatly enhanced by the adsorbent; the value of control PA is significantly less than that of the control CA.

The total mouse toxicity of the vaccines is characterized by the BWD$_7$ data. The regression lines are parallel but not identical (Tables 7,8). The lines of D and T differ significantly from the lines of DPA, TPA, and DTPA. The differences between the values of C, P, and CA are not statistically significant, but the value of PA is significantly less than the others. In all cases vaccines containing D were more toxic than those containing T, but the most toxic preparations were those containing both toxoid components (Fig. 10).

The number of deaths observed in the mouse toxicity test is shown in Table 9. Correlation be-

Figure 4. Mean body weight change of 72 mice after inoculation of adsorbed toxoids.

Table 5. Regression Analysis of the BWD_{5-2} Data Obtained with Different Vaccines

Vaccine	A + B × T	S.E. of A	S.E. of B	Regr. SSq	D.F.	Lin. F	D.F.	Sign.
D	3.16 − 0.05 × T	0.54	0.25	3.3084	13	1.3494	1/138	NS
T	3.36 − 0.06 × T	0.54	0.25	2.4222	13	0.0320	1/138	NS
DP	0.90 + 0.29 × T	0.54	0.25	6.6233	13	0.3301	1/138	NS
TP	0.80 + 0.26 × T	0.54	0.25	7.9893	13	0.6912	1/138	NS
DTP	0.78 + 0.45 × T	0.49	0.23	13.3417	16	1.4163	1/138	NS
DA	3.34 + 0.13 × T	0.49	0.23	5.7636	16	1.6320	1/138	NS
TA	3.81 + 0.03 × T	0.49	0.23	5.8639	16	0.0003	1/138	NS
DPA	0.55 + 0.14 × T	0.49	0.23	11.2490	16	0.0110	1/138	NS
TPA	0.87 + 0.16 × T	0.49	0.23	18.7249	16	0.0287	1/138	NS
DTPA	0.40 + 0.22 × T	0.49	0.23	13.9078	16	0.6953	1/138	NS

F test for parallelism: 0.44   D.F.: 9/138   P5% = NS   F test for identity: 39.73   D.F.: 9/138   P5% = S   Common regression coefficient: 0.16   Its S.E.: 0.07
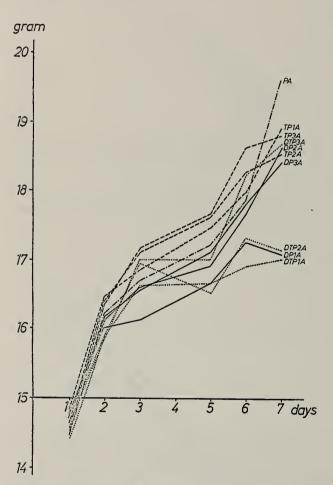
Figure 5. Mean body weight change of 72 mice after inoculation of adsorbed combined vaccines.

Table 6. Gabriel's Multiple Comparison Test of Treatment Means Computed From the BWD_{5-2} Data

Vaccine	Order	Order, Minimum	Order, Maximum
D	4	1	4
T	3	1	4
DP	6	5	10
TP	7	5	10
DTP	5	5	10
DA	2	1	4
TA	1	1	4
DPA	10	5	10
TPA	8	5	10
DTPA	9	5	10

Figure 6. Regression lines computed from the BWD_1 data in the mouse toxicity test.

toxicity, but it has never killed a mouse. Similarly, the early toxicity of higher doses of diphtheria toxoid is statistically significant and it does not kill mice either. Deaths occurred only in those groups that had been injected with pertussis vaccines or with combined vaccines containing a pertussis component. The lethal effect of pertussis vaccine is enhanced mainly by the adsorbent but by the toxoids as well. The lethality-enhancing

Table 7. Regression Analysis of the BWD₇ Data Obtained with Different Vaccines

Vaccine	A + B × T	S.E. of A	S.E. of B	Regr. SSq	D.F.	Lin. F	D.F.	Sign.
D	5.61 + 0.56 × T	0.87	0.40	26.3098	16	0.0305	1/150	NS
T	7.91 − 0.13 × T	0.87	0.40	9.4898	16	0.0317	1/150	NS
DP	2.53 + 1.21 × T	0.87	0.40	18.3667	16	1.6085	1/150	NS
TP	4.26 + 0.41 × T	0.87	0.40	25.1315	16	0.6198	1/150	NS
DTP	3.64 + 0.62 × T	0.87	0.40	29.2772	16	1.3789	1/150	NS
DA	5.43 + 0.44 × T	0.87	0.40	8.0943	16	0.6324	1/150	NS
TA	6.87 + 0.15 × T	0.87	0.40	3.5737	16	0.1216	1/150	NS
DPA	1.25 + 0.66 × T	0.87	0.40	34.1945	16	1.5846	1/150	NS
TPA	3.31 − 0.02 × T	0.87	0.40	56.5277	16	0.1284	1/150	NS
DTPA	0.52 + 0.82 × T	0.87	0.40	95.3066	16	1.1929	1/150	NS

F test for parallelism: 0.98 D.F.: 9/150 P5% = NS F test for identity: 34.29 D.F.: 9/150 P5% = S Common regression coefficient: 0.47 Its S.E.: 0.13

Table 8. Gabriel's Multiple Comparison Test of Treatment Means Computed from the BWD₇ Data

Vaccine	Order	Order, Minimum	Order, Maximum
D	3	1	5
T	1	1	4
DP	6	4	8
TP	5	3	7
DTP	7	4	8
DA	4	1	7
TA	2	1	4
DPA	9	8	10
TPA	8	6	10
DTPA	10	8	10
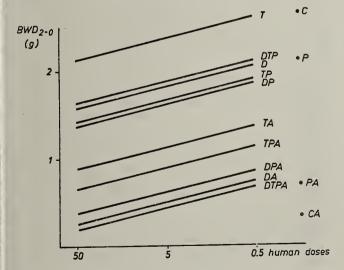
Figure 7. Regression lines computed from the BWD₂ data in the mouse toxicity test.

Figure 8. Regression lines computed from the BWD₃ data in the mouse toxicity test.

effect of diphtheria toxoid is slightly stronger than that of tetanus toxoid.

DISCUSSION

Besides regression analysis and analysis of variance for the evaluation of the experimental data obtained in mouse toxicity tests, Gabriel's multiple comparison test was introduced. The advantages of this method over the determination of absolute or relative body weight gain of mice are as follows:

1. By the analysis of the growth rate of uninjected mice, the homogeneity of the mouse colony can be estimated;

2. The analysis of growth rate of mice injected with pertussis or DTP vaccines gives information on the quantity of the toxic antigens and/or the sensitivity of the mouse strain;

3. By Gabriel's multiple comparison test the toxicity of phase products, pertussis vaccines, and

DTP vaccines can be compared. The sensitivity of different mouse strains can also be compared. The method can be used to select an appropriate mouse strain for the toxicity test and to check the sensitivity of the strain used.

Since the type of mouse strain used significantly influences the weight gain test, investigators often feel that they are assaying the mice, rather than the vaccines. Despite controversy over the mouse toxicity test, our results allow us to conclude that regression analysis and analysis of variance of dose-response functions, followed by Gabriel's multiple comparison regression coefficients and treatment means, is a correct statistical method for evaluating weight gain test data.

CONCLUSIONS

1. The early, late, and total mouse toxicities are characterized by data obtained from BWD_1, BWD_{5-2}, and BWD_7, respectively.

2. Aluminum phosphate, 1.5 mg, as adsorbent caused strong, early toxicity. The diphtheria toxoid also had an early toxic effect, but this effect could be demonstrated only with doses of 100–1,000 Lf per mouse. The adsorbent and toxoids, given in the same doses, had no lethal effect.

Figure 9. Regression lines computed from the BWD_{5-2} data in the mouse toxicity test.
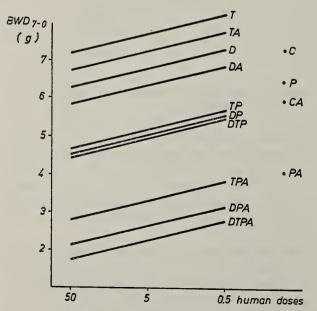
Figure 10. Regression lines computed from the BWD_7 data in the mouse toxicity test.

Table 9. Number of Deaths Observed in Mouse Toxicity Test

Treatment	Number of dead mice on day							Deaths/Total number of mice
	1	2	3	4	5	6	7	
C								0/72
CA								0/72
P					1	1	1	3/72
PA					6	8	6	20/72
D1								0/72
D2								0/72
D3								0/72
T1								0/72
T2								0/72
T3								0/72
DP1					2	1	7	10/72
DP2					2		2	4/72
DP3			1	2	2	1	4	10/72
TP1					1	4	3	8/72
TP2					3	2	2	7/72
TP3					1	2	1	4/72
DTP1					6	3	4	13/72
DTP2			1	2	3	2	1	9/72
DTP3			3	1	1	1	2	8/72
D1A								0/72
D2A								0/72
D3A								0/72
T1A								0/72
T2A								0/72
T3A								0/72
DP1A	1	6	3	2	6	3		21/72
DP2A		2	2	3	7	11		25/72
DP3A	1			5	6			12/72
TP1A		3	5	6	8	1		23/72
TP2A		3	5	5	3	4		20/72
TP3A		1		5	6			12/72
DTP1A		3	5	5	6	8		27/72
DTP2A		3	3	4	12	3		25/72
DTP3A		3	4	4	9	2		22/72

3. Aluminum phosphate gel, which had no late toxicity itself, significantly enhanced that of the pertussis vaccine.

4. Our experimental data, especially the data of BWD_7, show that diphtheria toxoid was more toxic than tetanus toxoid and that the combined vaccines containing diphtheria toxoid were more toxic than the corresponding preparations containing tetanus toxoid. It is probable that besides the toxic effect of adsorbent, the toxicity of diphtheria toxoid is due to the additional toxicity of the combined vaccines. This toxic effect was not due to reversion of diphtheria toxoid to toxin; according to the data of BWD_7, the diphtheria toxoid was not toxic itself, but it probably enhanced the effect of LPF. This characteristic can be attributed to the strong immunological adjuvant effect of the cell wall of *C. diphtheriae* P.W.8 (24,25), since the diphtheria toxoid may contain cell wall antigens after concentration and partial purification.

REFERENCES

1. Pittman, M. Influence of preservatives, of heat, and of irradiation on mouse protective activity and detoxification of pertussis vaccine. J. Immunol. 69:201–216; 1952.
2. Cohen, H. H. Development of pertussis vaccine production and control in the National Institute of Public Health in the Netherlands during the years 1950–1962. Antonie van Leeuwenhoek 29:183–210; 1963.
3. Pittman, M.; Cox, C. B. Pertussis vaccine testing for freedom-from-toxicity. Appl. Microbiol. 13:447–456; 1965.
4. Muggleton, P. W. Vaccines against pertussis. Public Health. 81:252–264; 1967.
5. Someya, S. Studies on the prophylactic effect and untoward reactions of pertussis-diphtheria combined vaccine. V. Relationship between the results from field trials and laboratory tests. Bull. Inst. Public Health. 16:23–32; 1967.
6. Gardner, R.; Bohner, H.; Pittman, M. Reactivity of germ-free and conventional mice to pertussis vaccine. Prog. Immunobiol. Stand. 3:315–318; 1969.
7. Ramshorst, J. D. van. Toxicity control of pertussis vaccines by the mouse weight gain test. Prog. Immunobiol. Stand. 3:324–326; 1969.
8. Barnes, J. M.; Hilton, M. L. The value of the mouse toxicity test for screening pertussis vaccine. International Symposium on Pertussis, Bilthoven, 1969. Symp. Series Immunobiol. Stand. 13:180–184; 1970.
9. Kindt, H. Pertussis toxicity testing of monovalent and combined vaccines. International Symposium on Pertussis, Bilthoven, 1969. Symp. Series Immunobiol. Stand. 13:185–189; 1970.
10. Pittman, M. *Bordetella pertussis*—bacterial and host factors in the pathogenesis and prevention of whooping-cough. Mudd, S., ed. Infectious agents and host reactions. Philadelphia: W. B. Saunders Co.; 1970:239–270.
11. Csizér, Z.; Joó, I.; Réthy, L.; Juhász, V. P. Laboratory testing of combined diphtheria-tetanus-pertussis-typhoid vaccines. Prog. Immunobiol. Stand. 5:394–398; 1972.
12. Ishida, S. Characterization of the body weight-decreasing toxicities in mice by the lymphocytosis-promoting factor and the heat-labile toxin of *B. pertussis* and endotoxin. Jpn. J. Med. Sci. Biol. 21:115–135; 1968.
13. Kurokawa, M.; Ishida, S.; Iwasa, S.; Asakawa, S.; Kurakutsa, K. Attempts at analysis of toxicity of pertussis vaccine. I. Body weight-decreasing toxicity in mice. Jpn. J. Med. Sci. Biol. 21:137–153; 1968.
14. Maróczi, J.; Ráskai, M. Contributions to toxin production of *Cl. tetani* in beef-heart broth digested with pepsin and trypsin. Ann. Immunol. Hung. 10:95–100; 1967.
15. Pusztai, Zs.; Joó, I.; Kiss, I. Some observations on the growth of *Bordetella pertussis* in liquid shaken cultures. Pathol. Microbiol. 23:192–200; 1960.
16. Surján, M. The large-scale production of tetanus toxoid. Schweiz. Z. Allg. Bakteriol. Pathol. 19:311–317; 1956.
17. Csizér, Z.; Joó, I. Large-scale production of pertussis vaccine by serial subculture technique. Zentralbl. Bakteriol. (Orig. A) 221:59–64; 1972.
18. Surján, M.; Richter, P.; Réthy, L. Purification of diphtheria and tetanus toxoids by trichloroacetic acid. Acta Microbiol. Acad. Sci. Hung. 1:339–348; 1954.
19. Réthy, L.; Kiss, I.; Elek, G.; Koós, R. Purification of the diphtheria toxoid by different chemical methods. Chemical and immunological characteristics of the purified antigens. Ann. Immunol. Hung. 6:25–33; 1963.
20. Pusztai, Zs.; Joó, I.; Juhász, P. V. Immunogenic properties of liquid shaken *Bordetella pertussis* cultures. Z. Immunit. Forsch. 122:278–290; 1962.
21. Finney, D. J. Statistical method in biological assay, 2d edition. London: Charles Griffin; 1971.
22. World Health Organization Expert Committee on Biological Standardization. Requirements for pertussis vaccine. WHO Tech. Rep. Ser. 274:25–40; 1964.
23. Gabriel, K. R. A procedure for testing the homogeneity of all sets of means in analysis of variance. Biometrics 20:458–477; 1964.
24. Azuma, I.; Kanetsuna, F.; Taniyama, T.; Yamamura, Y.; Hori, M.; Tanaka, Y. Adjuvant activity of mycobacterial fractions. I. Purification and in vivo adjuvant activity of cell wall skeletons of *Mycobacterium bovis* BCG, *Nocardia asteroides* 131 and *Corynebacterium diphtheriae* PW8. Biken J. 18:1–13; 1975.
25. Taniyama, T.; Watanabe, T.; Azuma, I.; Yamamura, Y. Adjuvant activity of mycobacterial fractions. II. In vitro adjuvant activity of cell walls of mycobacteria, nocardia and corynebacteria. Jpn. J. Microbiol. 18:415–426; 1976.

The Preservative Thimerosal as a Potentiator of Bowel Intussusception in Mice Injected with *Bordetella pertussis* Preparations

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ABSTRACT

A small percentage of adolescent mice of both sexes developed intussusceptions when injected intraperitoneally with thimerosal-preserved *Bordetella pertussis* suspensions. Injections by other routes (subcutaneous, intravenous, intrapleural) did not give intussusceptions, and needle trauma to the intestines was not important in this phenomenon. The condition did not occur in 5-week-old mice above 20 g body weight.

The responsible factor(s) was stable at 56°C, but was destroyed at 75°C. It shared some properties with Munoz's "pertussigen" and was absent from other bacterial genera tested. However, it also occurred in *B. bronchiseptica* and C-mode *B. pertussis*.

Thimerosal and *B. pertussis* interacted in some unknown way to produce the intussusceptions; neither was effective alone.

Three practical outcomes of these findings are indicated. First, in testing vaccines containing *B. pertussis*, only careful autopsies on all dead or sick mice will allow deaths from intussusception (in which thimerosal is implicated) to be differentiated from those solely attributable to "true" *B. pertussis* toxicity.

Second, the incidence of intussusceptions in routine vaccine control tests may be reduced substantially by keeping the thimerosal dose per mouse low.

Third, it is now possible to induce intussusceptions deliberately in adolescent mice, thus providing a model for studying the pathogenesis of this well-recognzed condition of both man and animals.

INTRODUCTION

For several years, *Bordetella pertussis* workers in several departments at Commonwealth Serum Laboratories (CSL) were plagued by unexpected deaths of mice on test. These deaths occurred only when mice were injected intraperitoneally with *B. pertussis* preparations. Autopsies showed that many of the unexpected deaths were due to bowel intussusception, generally in the ileocecal area, with accompanying bowel obstruction and often with intraluminal hemorrhage. Study of the literature revealed that intussusceptions were recorded in laboratory animals but were rare and that they also constituted an uncommon but serious surgical emergency for human infants (1-4).

In the course of 3 years we recorded approximately 180 intussusceptions from nearly 20,000 mice, partly from routine testing of experimental or production vaccine materials, and partly from experiments deliberately designed to investigate the intussusception phenomenon.

This is not solely a CSL-vaccine or CSL-mouse phenomenon. We have induced intussusceptions with both local and imported pertussis-containing vaccines and have learned that intussusceptions have been noted (but results not published) by Dr. Christensen in Denmark and by Dr. Cameron, when he was in England (personal communications, 1976). The results of some of our studies are the subject of this paper.

MATERIALS AND METHODS

Mice used were from our closed, random-bred colony established over 40 years ago from a Swiss white mouse strain. These mice were selected within a 5 g weight range at the start of each experiment, and from within an overall weight range of 8-15 g, except where the effect of mouse size was being investigated. Thimerosal or sodium ethylmercurithiosalicylate was supplied under Eli Lilly's trade name Merthiolate.

Bacterial Cultures

Heat-killed and thimerosal-preserved *Salmonella typhi* and *Vibrio cholerae* suspensions and live *Bordetella pertussis* suspensions were provided by the Aerobic Vaccines Production Department. The Bacteriology Research Department provided phenol-preserved *Salmonella pullorum* and *Clostridium novyi* suspensions, which were subsequently centrifuged, washed free of phenol, and resuspended in phosphate-buffered saline with 0.01% thimerosal. *Bordetella bronchiseptica*, *Bordetella parapertussis*, and some *B. pertussis* cultures were grown on Cohen and Wheeler Blood Agar (5) and harvested in thimerosal-phosphate-saline. Phenotypically "rough" (C-mode) *B. pertussis* was grown on Lacey's high-magnesium blood agar medium (6).

Injection Techniques

Intraperitoneal, intravenous, and subcutaneous injections were given by recognized procedures. Intrapleural injections were made through the intercostal space while the mouse was restrained in a normal standing position on a bench. A short 26-gauge needle was inserted into the dorsal area of the intercostal space near the vertebrae at about the 8–10th rib so as to enter the pleural cavity and avoid puncturing the heart or lungs. The injection volume was kept low to reduce respiratory embarrassment.

For intraperitoneal injection, a mouse was anesthetized with ether and taped down on its back, after which its abdomen was swabbed with 70% alcohol. Anesthesia was maintained by means of an ether pad in a tiny beaker held over its nose. To avoid needle trauma to the intestines, the operator picked up a "tent" of midline abdominal skin, about a third of the way between the base of the sternum and the pubis, and with sterile scissors removed a 2–4 mm patch of skin, leaving the abdominal musculature intact. This gave a semitransparent "window," which was crossed by the lower margin of the liver. The injection needle, with loaded syringe attached, was inserted through the abdominal wall over the liver and the test material discharged on the surface of the liver. The skin wound was then closed with a mattress suture and the mouse was returned to a cage with clean sawdust. Control mice were injected by inserting the needle over the intestines, thus exposing them to possible needle trauma. All animals that died were autopsied to ascertain the cause of death. Survivors were sacrificed for autopsy after 10 days.

RESULTS

Preliminary Observations

Most intussusceptions occurred within 6 days of intraperitoneal injection. Some occurred within 24 hours, but the peak occurred at day 5 (Fig. 1).

Figures 2–5 show the viscera of a normal mouse contrasted with those of an animal suffering from an intussusception. The latter was one that died on test unexpectedly. It is obviously abnormal with grossly distended intestines and a black, sausage-shaped area, which turned out to be the cecum and nearby gut, filled with about 2 inches of invaginated small intestine as well as a blood clot. This is the intussusception and the site of the bowel obstruction that killed the animal. There is much blood in the colon and rectum, but it has come from the ileo-cecal area, where the intussusception is located.

Range of Organisms Able to Produce Intussusceptions in Mice

Table 1 shows that large doses of *Bordetella bronchiseptica* can also cause intussusceptions, but that *B. pertussis* was much more active. Large doses of C-mode *B. pertussis* also produced a few intussusceptions. *B. parapertussis* had no activity in the limited number of animals studied. There was no sickness or death in control mice, so presumably they had no intussusceptions.

Other bacteria, both Gram-positive and Gram-negative, injected with 0.01% thimerosal failed, even in toxic amounts, to cause intussusception (Table 1). Thus intussusceptogenic activity seems to be restricted to *B. pertussis* and *B. bronchiseptica*.
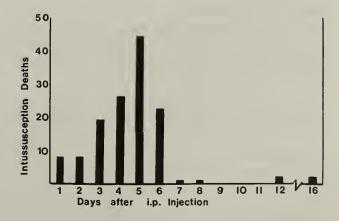
Figure 1. Times of death of mice from intussusception (horizontal axis) versus numbers of mice which died (vertical axis).

Table 1. Comparison of Various Killed Bacteria, Horse Blood, and Thimerosal-Saline for Intussusception-Inducing Capacity in Mice[a]

Cells	Dose per Mouse ($\times 10^9$ organisms)	Deaths	Intussusceptions/ Autopsies
B. pertussis	1.25–80	70/200	7/70 (3.5%)
B. bronchiseptica	100–200	12/80	3/11 (4.1%)
B. parapertussis	50–100	3/40	0/3
C. novyi	0.1–20	50/50	0/20
E. insidiosa	0.1–20	27/50	0/26
S. pullorum	2–30	22/50	0/22
S. typhi	2.5–40	18/50	0/18
V. cholerae	5–80	30/50	0/21
Horse blood	0.125–2.0 ml	0/50	—
Thimerosal-saline	1.0–2.0 ml	0/20	—

[a] All injections given intraperitoneally

Heat Stability of Intussusceptogenic Factor (IF)

B. pertussis suspensions were treated for 30 minutes at the various temperatures shown in Table 2 and tested for IF activity. All mice that became sick or died were autopsied. As Table 2 shows, there were many deaths in mice given unheated B. pertussis. Heating largely eliminated these "toxic" deaths. Intussusceptions were found only in animals given the unheated or 56°-heated materials. No intussusceptions and practically no deaths from any cause occurred in mice given materials heated at 75° C. Combining the unheated and 56° results, we had seven intussusceptions in 200 mice, compared to no intussusceptions in 200 mice in the combined 75° and 100° group. This difference is statistically significant (p = 0.74%, by Fisher's Exact Test).

Role of Injection Route

Only the intraperitoneal route was effective; subcutaneous, intravenous, or intrapleural injections did not give rise to intussusceptions (Table 3).

Role of Needle Trauma to the Bowel

Using the surgical technique described, we squirted an experimental vaccine of high IF activity directly on the intestines without touching them with a needle. We still found intussusceptions (3 of 12 mice injected over the liver, against 2 of 5 mice in which the intestines were exposed to possible needle trauma). Thus trauma to the bowel by the

Figure 2. Autopsy on normal mouse, showing abdominal viscera.

Table 2. Heat-Lability of Intussusception-Producing Factor

Cells Injected	Heat Treatment	Dose per Mouse ($\times 10^9$ organisms)	Deaths (total)	Intussusceptions/ Autopsies
B. pertussis (phase I)	4°	1.25–80	66/100	5/66 (5%)
	56°	2.5–40	4/100	2/4 (2%)
	75°	5–80	1/100	0/1 (0%)
	100°	5–80	0/100	—
B. pertussis, C-mode (phenotypically "rough")	56°	1.6–10	7/217	3/7 (1.4%)

Table 3. Effect of Route of Injection on Production of Intussusceptions

Injection Route	B. pertussis per Mouse ($\times 10^9$ organisms)	Thimerosal per Mouse (μg)	Intussusceptions/Autopsies		
Intraperitoneal	1.25–40	6.25–100	2/100	4/130	
	25–100	50	2/30		
Intravenous	1.25–40	6.25–100	0/60	0/120	0/180
	25–100	50	0/60		
Subcutaneous	25–100	50	0/30		
Intrathoracic	25–100	50	0/30		

injection needle was not required for the induction of intussusceptions.

Effect of Size and Sex of Mouse

In the course of normal pertussis work it became apparent that both sexes were affected, but small mice were more susceptible to intussusceptions than large mice. Some results demonstrating this are shown in Table 4.

Effect of Thimerosal

A clue to the involvement of thimerosal in the induction of intussusceptions in the mouse came from pertussis toxicity and potency tests in some storage stability experiments on certain experimental vaccines. We noted a different incidence of intussusceptions, according to whether the vaccine diluent contained thimerosal (Table 5).

Nelson and Gottshall (7) had observed that thimerosal potentiated the toxicity of intraperitoneally administered pertussis vaccines, so we investigated the effect of thimerosal in our system and discovered that it potentiated the occurrence of intussusceptions in mice. Table 6 consolidates the results of many experiments.

Increasing the B. pertussis dose leads to increased mortality (or extreme illness) from intussusceptions. Similarly, as the dose of thimerosal increases, so do

Table 4. Effects of Sex and Size of Mouse on Incidence of Intussusceptions

Initial Weights of Mice (g)	Male	Female	Combined
8–16	11/446	8/538	19/984
16–24	1/416	0/32	1/448
Combined	12/862	8/570	20/1432

Combined results from five experiments; doses 2–52 $\times 10^9$ B. pertussis and 20–250 μg thimerosal per mouse.

the intussusceptions. Both factors are necessary. At the very highest doses of thimerosal, however, there were no intussusceptions at all. This anomaly cannot be explained at present, but it is reproducible.

DISCUSSION

A problem throughout this investigation was the low percentage of mice that developed intussusceptions. Increasing the dose of B. pertussis vaccine was only a partial remedy, because mice began to die from the other toxic components of the organisms.

We initially suspected that the IF was identical to Munoz's "pertussigen" (otherwise known as histamine-sensitizing factor, HSF) (8) because of the similar heat lability and the peaking of both histamine sensitivity and intussusception incidence about 5 days after dosing. Thimerosal-treated suspensions of bacterial species such as Salmonella typhi, S. pullorum, Vibrio cholerae, Erysipelothrix insidiosa, and Clostridium novyi, which are known to lack pertussigenlike activity (9), failed to produce intussusceptions. Furthermore, in unpublished work involving experimental pertussis vaccine in storage tests connected with another project, one of us (A.C.B.) noted that three vaccines with high HSF activities gave rise to 3/30, 2/30, and 2/30 intussusceptions in toxicity tests, whereas seven vaccines with low HSF activity produced 2 intussusceptions from 220 mice (p = 0.28%, by Fisher's Exact Test). Storage of these vaccines for some months at 23° C and 37° C caused loss of both histamine-sensitizing and intussusceptogenic activity.

Histamine-sensitization is best achieved by intravenous injection of pertussis vaccine, although other routes are effective (8). Our results show, however, an absolute requirement for the intraperitoneal

Table 5. Effect of Thimerosal in Diluent (Results = Percent Intussusceptions)

Diluent	Dates	No. of Tests	B. pertussis per Mouse					Totals
			NIL	0.1×10^9	0.4×10^9	1.6×10^9	10×10^9	
Thimerosal-saline	12/75–1/76	2	0	0	0.8	2.9	6.4	1.7
	1/76–7/76	2	0.8	0.6	0.8	1.5	4.7	1.1
Saline (No thimerosal)	1/76–7/76	2	0	—	—	—	0	0
	7/76–3/77	3	0	0	0	0	0.1	0.04

Thimerosal in undiluted vaccines and diluent = 0.01%.

Table 6. Consolidated Intussusception Data (Oct. 1975–April 1977): Effects of Thimerosal and B. pertussis Dosages (Results = Percent Intussusceptions)

B. pertussis $\times10^9$ organisms/mouse	Thimerosal (μg/mouse)			Totals
	0–19	20–40	50–500	
Nil–0.05	0	0	0.13	0.07
0.06–0.9	0	0	0.64	0.36
1–160	0.09	1.03	4.0	1.86
Totals	0.03	0.80	1.69	1.00

injection route if mice are to develop intussusceptions. Since we induced a few intussusceptions with C-mode *B. pertussis* (6) and with large doses of *B. bronchiseptica,* neither of which is known to possess pertussigen (7), the nature of the IF remains unclear, except that it is apparently neither the 56°-labile *B. pertussis* toxin nor the 100°-stable lipopolysaccharide.

Intestinal lesions, but not intussusceptions, following administration of pertussis-containing vaccines, have been reported for mice (10) and hamsters (11). We have also noted severe hemorrhagic intestinal lesions with swollen mesenteric lymph nodes (but few intussusceptions) in pertussis-immunized mice challenged intraperitoneally with a *B. pertussis*-thimerosal mixture. The lesions resembled those described for anaphylaxis in the mouse (12), but most deaths occurred 5–8 days after challenge, and some as late as 2 weeks (Blaskett and Walden, unpublished data). Such lesions possibly represent some kind of delayed hypersensitivity or auto-immune reaction, as there was a positive response to both *B. pertussis*- and thimerosal-sensitizing dose levels (again with the exception that the very

Figure 3. Autopsy on a mouse that died unexpectedly after receiving an intraperitoneal injection of *B. pertussis* vaccine preserved with thimerosal. Note the abnormal appearance, with distended intestines and sausage-shaped black portion (mid-left), which is actually the cecum. The latter contains invaginated ileum and a blood clot, with adhesions and bowel obstruction, and is becoming gangrenous.

Figure 4. Same normal mouse as in Figure 2. The stomach (mid-left) is partly covered by the dark spleen. Note the normal cecum (upper right) and rectum.

Figure 5. Same mouse as in Figure 3. The stomach (center) lies over a pale spleen. Note again the distended small intestine and the congested blood vessels. The pale, sphincterlike ring in the ileum, beside the black sausage-shaped cecum (top right), is actually the start of the intussusception, where the intestine has doubled in on itself and blocked the bowel. The cecum and upper colon contained about 4 cm of intestine. The lower colon and rectum are black from the blood clot caused by hemorrhage from the intussusception.

highest thimerosal dosage, 250 μg per mouse, gave the smallest number of reacting mice). What connection, if any, there is between this kind of reaction to pertussis vaccine and the induction of intussusception remains unclear.

Concerning thimerosal, we know that it is an excellent antiseptic, reputedly superior to all other common vaccine preservatives for maintaining high protective potency in single or combined whooping cough vaccines (13). How it interacts with *B. pertussis* cells to increase toxicity (7) or produce intussusceptions remains conjectural. It might be related to its known potential allergenicity (14) or to some nonimmunological attribute, such as the pharmacological activity of a possible breakdown product produced (15) during normal vaccine processing or storage. The ultimate mechanisms concerned in induction of intussusceptions, however, is obviously confined to the peritoneal cavity.

Laboratories testing vaccines containing *B. pertussis* in the presence of thimerosal should be aware that mouse deaths may occur from intussusceptions of the bowel. These deaths presumably should be disregarded in assessing the toxicity of a vaccine, but the nuisance of them may be largely avoided by keeping the thimerosal dosage below the critical level of about 20 μg per mouse and using the largest mice compatible with the test.

Finally, the *B. pertussis*-thimerosal system could provide an experimental model for the study of the pathogenesis of intussusception, which constitutes an uncommon but dangerous surgical emergency in humans and animals.

REFERENCES

1. Munro, K. M. H. Causes of death in mated female mice. Lab. Anim. 6:67–74; 1972.
2. Schoenbaum, M.; Klopfer, U.; Egyed, M. N. Spontaneous intussusception of the small intestine in guinea pigs. Lab. Anim. 6:327–330; 1972.
3. Pollock, W. B. Prolapse of invaginated colon through the anus in golden hamsters (Mesocricetus auratus). Lab. Anim. Sci. 25:334–336; 1975.
4. Weisbroth, S. H.; Scher, S. Fatal intussusception associated with intestinal coccidiosis (Eimeria perforans) in a rabbit. Lab. Anim. Sci. 25:79–81; 1975.
5. Blaskett, A. C.; Gulasekharam, J.; Fulton, L. C. The occurrence of Bordetella pertussis serotypes in Australia, 1950–1970. Med. J. Aust. i:781–784; 1971.
6. Lacey, B. W. Antigenic modulation of Bordetella pertussis. J. Hyg. (Camb.) 58:57–93; 1960.
7. Nelson, E. A.; Gottshall, R. Y. Enhanced toxicity for mice of pertussis vaccines when preserved with merthiolate. Appl. Microbiol. 15:590–593; 1967.
8. Munoz, J. J.; Bergman, R. K. Bordetella pertussis: immunological and other biological activities. Rose, N., ed. New York, Basel: Marcel Dekker, Inc.; 1977: 47–107.
9. Munoz, J.; Bergman, R. K. Histamine-sensitizing factors from microbial agents, with special reference to Bordetella pertussis. Bacteriol. Rev. 32:103–126; 1968.
10. Culbertson, C. G.; Holmes, D. H.; Ensimger, P. W. Comparison of toxicity of pertussis preparations in conventional and germ-free mice and the presentation of the pathological bases for this observation. Pertussis Vaccine Symposium, N.I.H., Bethesda, Md., October 21, 1963; Proceedings I; 1963:153–166.
11. Zoltowska, A. Results of intensive immunization of rodents with the diphtheria-tetanus-pertussis vaccine. Arch. Immunol. Ther. Exp. 19:749–765; 1971.
12. Wilson, G. S.; Miles, A. A. Topley and Wilson's Principles of Bacteriology and Immunity, London: Edward Arnold; 1955:1300–1301.
13. Gardner, R. A.; Pittman, M. Relative stabilities of pertussis vaccine preserved with merthiolate, benzethonium chloride, or the parabens. Appl. Microbiol. 13:564–569; 1965.
14. Möller, H. Sensitizing aspects of preservatives. J. Biol. Stand. 3:241–245; 1975.
15. Davisson, E. O.; Powell, H. M.; MacFarlane, J. O.; Hodgson, R.; Stone, R. L.; Culbertson, C. G. The preservation of poliomyelitis vaccine with stabilized merthiolate. J. Lab. Clin. Med. 47:8–19; 1956.

Quantitative Immunoelectrophoretic Approach to the Characterization of *Bordetella pertussis* Antigens and Monitoring of Vaccine Production

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ABSTRACT

Water-soluble antigens from *Bordetella pertussis* were analyzed by quantitative crossed immunoelectrophoresis using sera from immunized rabbits.

Forty-nine different antigens have been demonstrated in each of the four strains of *B. pertussis* constituting the Danish vaccine. Three of the antigens have been further characterized: 1) a PAS-positive, heat-stable, *Bordetella* specific antigen which disappears upon addition of *Limulus* lysate; 2) an acid-precipitable fraction from the supernatant of *B. pertussis* cultures with lymphocytosis promoting and mitogenic activity; 3) an antigen crossreacting with several other Gram negative species. The antigenic compositions of X- and C-mode variants of one strain of *B. pertussis* have been compared, revealing that the C-mode variant lacked two of the antigens present in the X-mode. The various steps of pertussis vaccine production were analyzed: addition of formalin to the bacteria involved demonstrable loss of antigens, and it was shown that the batch of final vaccine tested contained one antigen originating from the Bordet-Gengou medium.

The antibody response in humans to *B. pertussis* antigens was evaluated, and precipitating antibodies against 5 of the 49 antigens were found in sera from children and adults.

MATERIALS AND METHODS

Antigen Preparations

Bordetella pertussis reference antigen (ref-ag). The four strains of *B. pertussis* used in the Danish vaccine (Statens Seruminstitut, strain numbers 3803, 3825, 3843, and 3860) were cultured separately on Bordet-Gengou medium for 72 hours at 35° C, washed three times in saline, suspended in water and sonicated (three 45-second exposures, ice-cooled, at 20,000 Hz/sec) using a Rapidis 300, 19 mm probe with a 9.5 mm tip). After centrifugation (48,000 × g, 60 minutes, 4° C), equal volumes of the supernatants were mixed and stored at −30° C. The colloid concentration (measured by refractometry using human IgG as a standard) was 11.5 g/l (5,6).

Bordetella pertussis X- and C-mode variants (X- and C-ag). Phase I *B. pertussis* 18334, grown on Bordet-Gengou medium at 35° C for 72 hours, was suspended in modified Hornibrook medium (7,8) containing 5 g NaCl/l to give X-mode cells and 5 g MgSO$_4$/l to obtain the C-mode. The cells were incubated at 35° C for 72 hours, then sonicated, centrifuged, and mixed as described. The colloid concentration was 12.5 g/l.

Bordetella pertussis vaccine antigens (vacc-ag). Samples of the four strains were obtained during the production of vaccine (Serum and Vaccine Department, Statens Seruminstitut). The culture supernatants (Table 1) were examined after filtration with 0.22 μm Millipore^R and the antigens of the cells were examined after the washing-sonication-centrifugation procedures described.

Bordet-Gengou medium antigens (med-ag). Bordet-Gengou medium, as used for culture of the vaccine strains (containing potato agar–200 ml, beef broth–250 ml, defibrinated horseblood–200 ml, and activated charcoal–1.4 g), was sonicated and centrifuged as described and the supernatant was used as an antigen. The colloid concentration was 14.4 g/l.

Fractions of Bordetella pertussis with lymphocytosis-promoting and mitogenic activity. The methods of purification and the biological activities of these fractions are described elsewhere (9).

Rabbit Antisera (ref -ab, X- and C-ab, med-ab)

Separate antisera were prepared against each of the antigen preparations 1,2, and 4 (ref-ag, X- and C-ag, med-ag). Three rabbits were immunized intradermally with the appropriate antigen preparation in Freund's Incomplete Adjuvant, following the immunization and bleeding schedule of Harboe and Ingild (10); i.e., each animal received 100 μl

antigen per immunization. IgG and IgA were puri-
fied and concentrated. The first bleeding was used
for pilot experiments, and immunoglobulins from
subsequent bleedings were pooled, since immuno-
electrophoretic analysis revealed qualitatively sim-
ilar antibody patterns. All the rabbits were immu-
nized for at least 6 months. Preimmunization sera
from all the rabbits contained precipitating anti-
bodies against two of the *B. pertussis* antigens. (5).

Limulus Amoebocyte Lysate (LAL)

Blood from *Limulus polyphemus* was collected
without anticoagulant. The cells were sedimented
by centrifugation, disintegrated in a Micro-Dis-
membrator (Braun, West Germany), suspended in
pyrogen-free distilled water 1:4(w/v), centrifuged
(48,000 × g for 1 hour) and the supernatant used
as LAL. *B. pertussis* ref-ag was incubated with
LAL in several different proportions (v/v) for 4
hours at 37° C, the mixtures were centrifuged
(48,000 × g for 1 hour), and both the supernatant
and the residue were examined in the immuno-
electrophoretic experiments. The sensitivity of the
LAL test (L. Baek et al; in preparation) was 10^{-12}
g of *E. coli* endotoxin per ml [*E. coli* 026:B6 (Difco),
Westphal]. LAL incubated with pyrogen-free dis-
tilled water and *E. coli* endotoxin served as negative
and positive controls in the incubation experiments.

Immunoelectrophoretic Methods

Immunoelectrophoretic analysis of *B. pertussis*
antigens was performed by crossed immunoelectro-
phoresis on 5 × 5 or 10 × 10 cm glass plates using
1% agarose (Indubiose A–37) in Tris-barbital buf-
fer, pH = 8.6, ionic strength = 0.02. First dimension
electrophoreses were run for 30 minutes at 12° C,
10 volts/cm², second dimension electrophoreses for
18 hours, 2 volts/cm². Coomassie Brilliant Blue-R
was used to stain the precipitates (1,5,6,11). Optimal
resolution was obtained if 2 μl antigen was used
in the first dimension electrophoresis and 20 μl
rabbit antiserum per cm² in the second dimension
gel. The various antigen-preparations were ana-
lyzed and compared by the following methods.

1. Crossed immunoelectrophoresis against the
corresponding rabbit antiserum.
2. Crossed-line electrophoresis (5,6,12) with the
antigen in question incorporated in an intermediate
gel between first and second dimension of antigen
and corresponding rabbit antiserum (absorption
of antibodies in situ). The intermediate gel con-
tained 20 μl, 40 μl or 80 μl per cm² of the antigen
preparation.
3. Tandem-crossed immunoelectrophoresis (5,6,
13).

Human serum-antibodies against *B. pertussis* an-
tigens were identified and quantitated by means of:
4. Crossed immunoelectrophoresis with human
serum incorporated in an intermediate gel (40μl/
cm²), using *B. pertussis* reference antigen vs. rabbit
antiserum as a reference (2,5,14).

The sensitivities and the analytical variations of
the methods have been described (5,6).

Human Sera

Sera from healthy adults, children undergoing
the normal vaccination schedule, and children with
whooping cough were examined as described pre-
viously (5).

RESULTS

The crossed immunoelectrophoretic pattern of
the *B. pertussis* reference antigen preparation run
against corresponding rabbit antiserum is shown
in Figure 1. Forty-nine precipitation lines were
detected, with 44 antigens migrating toward the
anode and 5 toward the cathode. All precipitates
have been enumerated (5). Different approaches to
the characterization of the individual antigens were
attempted. Antigen number 26 (Fig. 1) was the
only one that could be stained with the periodic
acid Schiff (PAS) agent; it is heat stable, and anti-
bodies against it could be adsorbed by whole heat-
killed *B. pertussis* cells. Furthermore, this antigen
disappeared upon incubation with LAL. Absorp-
tion experiments showed that this antigen is *Bor-
detella*-specific, crossreacting only with *B. bronchi-
septica*. Antigen number 11 (Fig. 1) was shown to·

Figure 1. Crossed immunoelectrophoretic pat-
tern of a sonicated preparation of the Dan-
ish vaccine strains of *B. pertussis* (ref-ag)
run against corresponding rabbit antiserum
(ref-ab). *First dimension:* Anode to the right.
Second dimension: Anode at the top. 10 ×
10 cm glass plate. The bar indicates 1 cm.
Arrows indicate Antigen 11, a crossreactive
antigen, and antigen 26, a *Bordetella*-specific,
heat-stable surface antigen.

crossreact with several other, mainly Gram-negative, bacterial species (15).

The tandem-crossed immunoelectrophoretic experiments in Figure 2 showed that an acid-precipitated fraction with lymphocytosis-promoting and
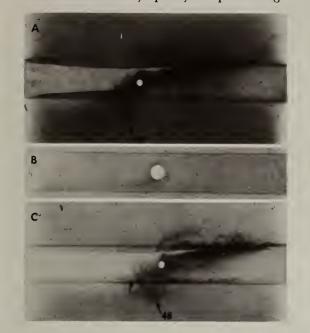
Figure 2. Tandem-crossed immunoelectrophoresis comparing a fraction with lymphocytosis promoting and mitogenic activity with antigens of the sonicated preparation of the Danish *B. pertussis* vaccine strains. A. Crossed immunoelectrophoresis of ref-ag run against ref-ab, as in Figure 1. B. Crossed immunoelectrophoresis of an acid-precipitated fraction (from the culture supernatant of one of the Danish vaccine strains) with lymphocytosis-promoting activity, run against ref-ab. C. Tandem-crossed immunoelectrophoresis of the fraction used in B (left well) and ref-ag (right well) run against ref-ab. Arrow indicates reaction of identity between antigen 48 of the vaccine strains and the acid-precipitated fraction. Technical detail as in Figure 1.

mitogenic activity is immunologically identical with one of the antigens of the Danish *B. pertussis* vaccine strains.

Figure 3 illustrates the few qualitative antigenic differences between X- and C-mode *B. pertussis* preparations as revealed by crossed-line immunoelectrophoresis. Only two antigens of the complex antigenic patterns seem specific for the X-mode variant.

Antigen change or loss of antigens during production of pertussis vaccine was investigated in four samples obtained at different steps during the preparation procedure as shown in Table 1. An example of the comparative experiments for one of the strains is shown in Figure 4. It was demonstrated that some of the antigens present in the initial preparations were not detectable in the antigen preparations obtained from the steps following the killing of the bacteria with formalin.

The detection of antigens originating from the culture medium used in vaccine production is illustrated in Figure 5. The results were similar for all four strains: one distinct antigen originating from the culture medium was present in the first culture supernatants, but had disappeared in the subsequent supernatants. Another antigen from the medium was consistently present in all batches investigated, including the final vaccine preparation. This antigen is present in horse blood, which is used in Bordet-Gengou medium (unpublished results).

Examples of the antibody patterns in human sera and commercial γ-globulin are shown in Figure 6. All sera examined contained precipitating antibodies against *B. pertussis* antigens. Antibodies
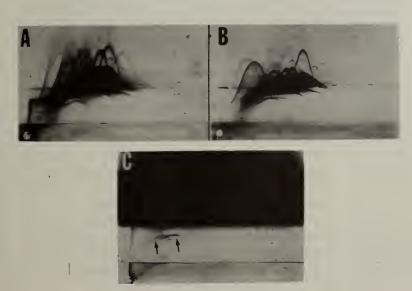
Figure 3. Crossed-line immunoelectrophoresis of sonicated preparations of X- and C-mode variants of *B. pertussis* strain 18334 (X- and C-ag). A. Crossed immunoelectrophoresis of a sonicated preparation of strain 18334 X-mode (X-ag) run against corresponding rabbit antiserum = X- and C-ab. (Obtained from rabbits immunized with a mixture of X- and C-mode preparations.) Saline in the intermediate gel. B. Crossed immunoelectrophoresis of C-ag run against X- and C-ab. Saline in the intermediate gel. C. Crossed-line immunoelectrophoresis of X-ag in the well and C-ag incorporated in the intermediate gel (40 μl/cm^2). X- and C-ab in the second-dimension gel. Two precipitation lines are seen in the intermediate gel (arrows), indicating that the C-mode variant did not absorb rabbit antibodies against all the antigens present in the X-mode variant. Technical detail as in Figure 1 (5 × 5 cm plates).
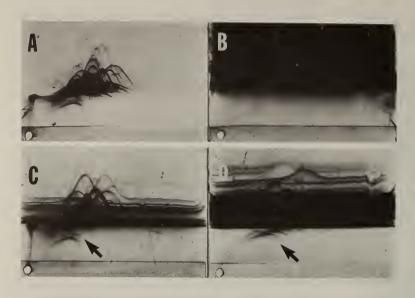
Figure 4. Investigation of antigenic loss during the preparation of pertussis vaccine. A. Control: antigen batch 1 (after the first culture, see Table 1) in the well. Saline in the intermediate gel; ref-ab in the second-dimension gel. B. Crossed-line immunoelectrophoresis of antigen batch 1 in the well and antigen batch 2 (after the second culture; see Table 1) in the intermediate gel; ref-ab in the second-dimension gel. All of the precipitates of the reference pattern have been removed or elevated and corresponding straight precipitation lines showing reaction of identity with elevated reference precipitates are seen in the second-dimension gel. Conclusion: No qualitative differences between batch 1 and batch 2. C. Crossed-line immunoelectrophoresis of antigen batch 1 in the well and antigen batch 3 (after the formalin killing; see Table 1) in the intermediate gel, ref-ab in the second-dimension gel. A few precipitates (arrow) are seen in the intermediate gel, indicating that antigen batch 3 did not absorb rabbit antibodies against all the antigens present in antigen batch 1. Conclusion: Antigen batch 3 lacks antigens compared with antigen batch 1. D. Crossed-line immunoelectrophoresis of antigen batch 4 (final vaccine; see Table 1) in the intermediate gel. Ref-ab in the second-dimension gel. Explanation and conclusion same as for Figure 2C.

against a total of 5 of the 49 antigens were present, including antigens 11 and 26 (5). Antibody production was demonstrable during the course of vaccination; antibodies present before vaccination increased in titer (e.g., antigens 11 and 26) and new antibody specificities appeared (5). The presence of antibodies against the cathodic migrating antigens (e.g., the fraction with lymphocytosis-promoting and mitogenic activity) has not yet been investigated.

Table 1. Preparation of Pertussis Vaccine

Procedure	Antigen Batches Investigated
Freeze-dried *B. pertussis* strain	
↓	
Inoculation: B–G medium, 48 hours	
↓	
Harvest: broth	
↓ ←	Batch 1
Inoculation: B–G medium, 48 hours	
←	Batch 2
Harvest: 0.9% NaCl containing 0.1% formalin (0.04% formaldehyde)	
↓	
5 days, room temperature	
↓ ←	Batch 3
Centrifugation	
↓	
Suspension: 0.9% NaCl containing 0.01% Merthiolate	
↓ ←	Batch 4
Final vaccine	

DISCUSSION

This study shows that quantitative immunoelectrophoretic methods can be used to analyze several aspects of *B. pertussis* antigens. Because individual antigens are visualized, it is possible to correlate differences in biological activity between different preparations with presence or absence of particular antigens.

X- and C-mode variants of *B. pertussis* differ in biological properties (8,16). The antigenic deficiencies of C-mode variants found by crossed immunoelectrophoresis are similar to the reduction in number of polypeptide bands demonstrated by SDS-polyacrylamide gel electrophoresis of *B. pertussis* envelopes (7), so it should now be possible to correlate the biological deficiency of C-mode variants to the deficiency of individual antigens.

According to these results, quantitative immunoelectrophoretic methods are suitable tools for investigation of various aspects of vaccine production including 1) the antigenic composition of the
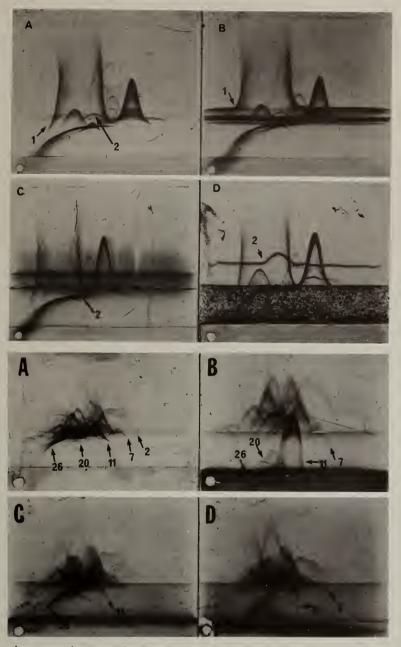
Figure 5. Search for antigens originating from the culture medium used in vaccine preparation. A. Control. Crossed immunoelectrophoretic pattern of sonicated Bordet-Gengou medium in the well run against corresponding rabbit antiserum in the second-dimension gel. Saline in the intermediate gel. B. Crossed-line immunoelectrophoresis of med-ag in the well against med-ab in the second dimension gel with *B. pertussis* antigen batch 1 supernatant (see Table 1) in the intermediate gel. One of the antigens of the reference pattern has been elevated (arrow) by the straight precipitation line showing reaction of identity. Conclusion: Medium antigen 1 is present in the *B. pertussis* supernatant. C–D. Crossed-line immunoelectrophoresis of med-ag in the well against med-ab in the second-dimension gel with *B. pertussis* antigen batches 2 and 4 (see Table 1) in the intermediate gel. One of the antigens of the reference pattern (arrow) has been elevated by the straight precipitation line showing reaction of identity. Conclusion: *Bordetella* antigen batches 2 and 4 contain med-ag No. 2.

Figure 6. Examples of crossed immunoelectrophoresis of sonicated *B. pertussis* reference antigen run against corresponding rabbit antiserum in the second-dimension gel, with an intermediate gel containing human serum. *Intermediate gels contain*: A. Saline (control). B. Human γ-globulin. Antibody specificities against ref-ag present in the γ-globulin preparation are indicated by arrows. C. Serum from a child before the first pertussis vaccination. Antibody specificities against ref-ag present in the serum are indicated by arrows. D. Serum from the same child after the third pertussis vaccination. Antibody specificities against ref-ag present in the serum are indicated by arrows. C–D. Interpretation: A rise in number and titers of precipitins is seen during the course of vaccination.

microorganisms used (5,6,15); 2) antigen change or loss during the production procedure; 3) presence of extraneous agents in the vaccine; and 4) the humoral immune response to the vaccine antigens.

Antigens lost or changed during vaccine production can be identified in quantitative immunoelectrophoresis, and a comparison can be made between differences in protective and toxic properties. The possibility that formalin treatment alters the extractability of certain antigens cannot be ruled out; the addition of formalin to the *B. pertussis* reference antigen preparation during vaccine production does not induce qualitative changes in

the immunoelectrophoretic pattern (unpublished observations).

The presence of an antigen from horse serum has been established in a batch of pertussis vaccine. Quantitative immunoelectrophoretic methods permit monitoring of changes in the preparation procedure to eliminate such antigens.

ACKNOWLEDGMENTS

REFERENCES

1. Axelsen, N. H. Analysis of human *Candida* precipitins by quantitative immunoelectrophoresis. Scand. J. Immunol. 5:177–189; 1976.
2. Høiby, N. *Pseudomonas aeruginosa* infection in cystic fibrosis. Acta. Pathol. Microbiol. Scand., Sect. C, Suppl. No. 262, 1977.
3. Bjerrum, O. J. Immunochemical investigation of membrane proteins. Biochim. Biophys. Acta 472:135–195; 1977.
4. Smyth, C. J.; Siegel, J.; Salton, M. R. J.; Owen, P. Immunochemical analysis of inner and outer membranes of *E. coli* by crossed immunoelectrophoresis. J. Bacteriol. 133:306–319; 1978.
5. Hertz, J. B.; Høiby, N.; Andersen, V.; Baekgaard, P. Crossed immunoelectrophoretic study of *B. pertussis* antigens and of corresponding antibodies in human sera. Acta Pathol. Microbiol. Scand., Sect. B. 84:386–394; 1976.
6. Høiby, N. Cross-reactions between *P. aeruginosa* and thirty-six other bacterial species. Scand. J. Immunol. 4: Suppl. 2, 187–196; 1975.
7. Parton, R.; Wardlaw, A. C. Cell-envelope proteins of *B. pertussis*. J. Med. Microbiol. 8:47–57; 1974.
8. Wardlaw, A. C.; Parton, R.; Hooker, M. J. Loss of protective antigen, histamine-sensitizing factor and envelope polypeptides in cultural variants of *B. pertussis*. J. Med. Microbiol. 9:89–99; 1975.
9. Andersen, V.; Hertz, J. B.; Nicolaisen, E. M.; Claësson, M.; Hansen, G. S.; Hansen, G. A.; Christensen, P. E. Properties of lymphocytosis promoting factor from *B. pertussis*. This conference.
10. Harboe, N.: Ingild, A. Immunization, isolation of immunoglobulins, estimation of antibody titer. Scand. J. Immunol. 2: Suppl. 1, 161–165; 1973.
11. Weeke, B. Crossed immunoelectrophoresis. Scand. J. Immunol. 2: Suppl. 1, 47–56; 1973.
12. Krøll, J. Crossed-line immunoelectrophoresis. Scand. J. Immunol. 2: Suppl. 1, 79–81; 1973.
13. Krøll, J. Tandem-crossed immunoelectrophoresis. Scand. J. Immunol. 2: Suppl. 1, 57–59; 1973.
14. Axelsen, N. H. Intermediate gel in crossed and in fused rocket immunoelectrophoresis. Scand. J. Immunol. 2: Suppl. 1, 71–77; 1973.
15. Høiby, N.; Hertz, J. B.; Andersen, V. Crossed-reactions between *B. pertussis* and twenty-eight other bacterial species. Acta Pathol. Microbiol. Scand., Sect. B. 84:395–400; 1976.
16. Wardlaw, A. C.; Parton, R. Changes in envelope proteins and correlation with biological activities in *B. pertussis*. This conference.

Selective Breeding to Establish a Standard Mouse for Pertussis Vaccine Bioassay
III. Characterization of HSFS/N Mice

K. P. Smith, C. T. Hansen, and C. R. Manclark

ABSTRACT

Two lines of mice were selectively bred for susceptibility and resistance to sensitization by the histamine-sensitizing factor (HSF) of *Bordetella pertussis* and were designated HSFS/N and HSFR/N respectively. Weanling mice were injected with a suspension of pertussis vaccine in saline and after 5 days were challenged with histamine diphosphate. A minimum of 12 offspring from the first two litters were tested from each mating pair in each generation. The selected parents from a third litter were brother × sister mated. After 20 generations of selection, the ability to be sensitized to HSF in the HSFS/N line had increased from 31% in the base generation to 70% and in the HSFR/N line had decreased from 31% to 0.5%. The estimates of the realized heritability were 32% in the HSFS/N line and 22% in the HSFR/N line. Immunizability with pertussis vaccine was measured after the 14th generation of selection. The mice from the HSFS/N line were found to be more immunizable than mice from the base population. After 18 generations of selection, immunizability was measured again, but the HSFS/N line was no longer more easily immunized than the base population. In each generation the reproductive performance of the two lines was measured. The reproductive indexes averaged over all generations were 4.0 for the HSFS/N strain and 3.8 for the HSFR/N strain. The HSFS/N and HSFR/N lines have been further characterized for 12 isozymes. As expected, after 20 generations of inbreeding both lines are isogenic. There were phenotypic differences between the lines for 2 of the 12 loci tested.

Introduction

The ideal animal for biological assays would be one for which the dose-response relationship is accurately predictable within a given dose range. In practice, however, the animal is often an unknown quantity except for age, sex, and strain. This is a further report on the attempts to develop a mouse strain with predictable and stable characteristics for the control testing of pertussis vaccine and other biologics. Hansen et al. (1), after eight generations of selective breeding, demonstrated that induction of histamine-sensitization by pertussis vaccine is a trait at least partly under genetic control. Our report deals with the genetic responses to selection for the histamine-sensitizing factor (HSF) in strains HSFS/N and HSFR/N after 20 generations of selection. The two strains are also characterized for histamine sensitizability, immunizability with pertussis vaccine, reproductive performance, and 12 biochemical markers.

Selection Procedures

From a base population of NIH Swiss Webster stock N:NIH(SW), two lines of mice were selectively bred for their susceptibility and resistance to sensitization by the histamine-sensitizing factor of *B. pertussis* and were designated HSFS/N and HSFR/N respectively. Weanling mice were injected intraperitoneally (i.p.) with one opacity unit (opu) of pertussis vaccine, lot 7b. After 5 days they were challenged with 100 mg of histamine diphosphate per kg of body-weight. Almost all deaths due to sensitization occurred within an hour. From the first two parities a minimum of 12 progeny of each mating pair were tested in each generation. The criterion for selection in each generation was based on the percentage of the progeny-deaths for each breeding pair relative to the mean percentage of deaths for all pairs of that generation for the sensitive and resistant lines respectively. Selected pairs for the next generation were usually taken from the third parity. In each generation the selected pairs were brother × sister mated.

Response to Selection

The response to selection for both the HSFS/N and HSFR/N strains after 20 generations of selection is shown in Figure 1. The sensitivity in the HSFS/N strain increased from 31% in the base generation to 70% in generation 20. The response
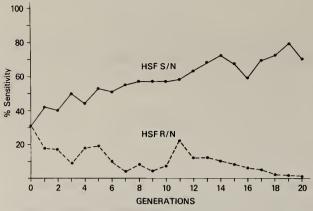
Figure 1. Response to selection for HSF.

Figure 2. Regression of response on selection differential.

has continued even in the later generations when genetic variation should be approaching fixation as a result of increased inbreeding. In the HSFR/N strain the sensitivity decreased to 0.5% in generation 20. The HSFR/N strain has essentially reached the limit of response at this dose level of immunization and challenge. The standard errors of the strain by generation-means ranged from 0.02 to 0.05%.

The realized heritability, estimated from the regression of the response on the selection differential, was 0.32 ± 0.02 in the HSFS/N and 0.22 ± 0.04 in the HSFR/N (Fig. 2). These heritabilities were estimated as twice the calculated regression coefficient, since the selections were based on measurements from progeny tests. These heritability estimates indicate that between 18 and 34% of the observed variation in histamine-sensitivity is due to genetic factors.

Reproductive Performance

The reproductive performance of each breeding pair in each generation was measured and included the following parameters: birthdate of the breeding pair, number of pups born, number of pups weaned, and date the breeding pair was retired. From these data a reproductive index was calculated for each breeding pair. The reproductive index for an individual breeding pair was defined as the total number of pups weaned from all litters, divided by the total number of 28-day periods the pair remained in the colony, i.e., the total number of 28-day periods between the birthdate of the pair and the date of retirement. The reproductive index is considered a more useful estimate of reproductive capacity than average litter size since it is a measure of total reproductive yield for a given period.

The average number of breeding pairs per gen-

eration was 25 for each strain. The average length of time a breeding pair remained in the colony was 207 days with a standard deviation of 65 days. The overall colony indexes for the 20 generations were 4.00 ± 0.07 and 3.80 ± 0.10 for the HSFS/N and the HSFR/N strains respectively. These values compare closely with colony indexes of other, high-yielding inbred strains maintained in the same facilities (2). Figure 3 shows that the indexes have decreased for both strains, which would be expected as a result of inbreeding depression. The HSFS/N strain has shown a large decrease in reproductive yield in the last two generations. It is not possible to determine at this time whether this is a permanent change due to increased inbreeding or a temporary result of unknown environmental factors.
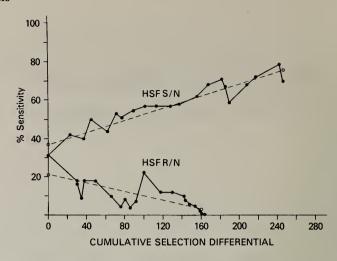
Histamine Sensitization

Since the breeding colony in any one generation was not large enough to carry out the necessary tests to characterize the strains, expansion colonies were established and their offspring were used for testing. The HSFS/N and HSFR/N strains were characterized between generations 11–14 by Manclark et al. (3). The HSFS/N strain has been further tested in generations 18–20. Weanling HSFS/N and HSFR/N mice from generations 11–14 were injected intraperitoneally with 0.5 ml of a saline suspension containing graded amounts of pertussis vaccine, lot 7b. HSFR/N mice were injected with doses containing 0.4 to 40 opu, HSFS/N 0.08 to 10 opu and N:NIH(SW) mice, used as a control, were injected with doses containing 0.04 to 25 opu. Weanling HSFS/N mice from generations 18–20 were injected with doses ranging from 0.1 to 2.5
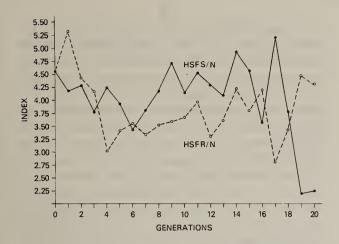
Figure 3. Reproductive index.

opu; doses for the N:NIH(SW) controls ranged from 1.5 to 6.0 opu. Control animals in each strain and sex were injected with 0.5 ml of saline. At the end of five days all mice were challenged intraperitoneally with 100 mg of histamine diphosphate per kg of body-weight. Median, histamine-sensitizing doses (HSD_{50}) were calculated by Wilson-Worcester analysis.

The HSD_{50} results are summarized in Tables 1 and 3, and the comparative ratios to the N:NIH(SW) are given in Tables 2 and 4. Only the slightest decrease in the HSD_{50} was observed for the HSFS/N females: from 0.51 to 0.50 opu for the measurements taken in generations 11–14 and generations 18–20 respectively. A slightly higher increase was observed, from 0.64 to 0.70 opu, for the HSFS/N males. After 14 generations of selective breeding, it takes 6.45 times more vaccine to sensitize the N:NIH(SW) female mice, compared with the HSFS/N females and 5.21 times more vaccine to sensitize the N:NIH(SW) males compared with the HSFS/N males. Similar relative values were observed for the HSFS/N mice after 18–20 generations.

N:NIH(SW) female mice were sensitized by one-third of the amount of vaccine (0.38) required for HSFR/N females. Similar results were observed for HSFR/N males compared with N:NIH(SW) males. The standard errors of the HSD_{50} values in Table 3 show not only that the HSD_{50} values have been reduced in the HSFS/N strain, but also that the variance has been reduced. The results from the assays for sensitivity to histamine give further evidence that through selective breeding two strains of mice have been developed which differ significantly in their response.

Table 2. Ratios of Histamine-Sensitizing Doses (HSD_{50}) for HSFS/N and HSFR/N Strains after Generations 11–14[a]

Strain	$\dfrac{HSD_{50}}{HSD_{50}}$
$\dfrac{\text{N:NIH(SW) } ♀}{\text{HSFS/N } ♀}$	$\dfrac{3.29}{0.51}=6.45$[b]
$\dfrac{\text{N:NIH(SW) } ♂}{\text{HSFS/N } ♂}$	$\dfrac{3.34}{0.64}=5.21$[b]
$\dfrac{\text{N:NIH(SW) } ♀}{\text{HSFR/N } ♀}$	$\dfrac{3.29}{8.55}=0.38$[b]
$\dfrac{\text{N:NIH(SW) } ♂}{\text{HSFR/N } ♂}$	$\dfrac{3.34}{4.97}=0.67$

[a] Ref. 3.
[b] $P<0.05$

Table 3. Determination of Median Histamine-Sensitizing Dose (HSD_{50}) After Generations 18–20[a]

Strain	Sex	HSD_{50} (opu)	Number of Mice
HSFS/N	♀	0.50 ± 0.08[a]	105
HSFS/N	♂	0.70 ± 0.09	110
N:NIH(SW)	♀	2.01 ± 0.33	104
N:NIH(SW)	♂	3.37 ± 0.75	102

[a] Standard error

Table 1. Determination of the Median Histamine-Sensitizing Dose (HSD_{50}) After Generations 11–14[a]

Strain	Sex	HSD_{50} (opu)	95% Confidence Limits	Number of Mice
HSFS/N	♀	0.51	0.49– 0.68	302
HSFS/N	♂	0.64	0.50– 0.82	300
HSFR/N	♀	8.55	6.70–10.92	335
HSFR/N	♂	4.97	3.51– 7.04	320
N:NIH(SW)	♀	3.29	1.98– 5.48	192
N:NIH(SW)	♂	3.34	2.09– 5.35	191

[a] Ref. 3.

Table 4. Ratios of Histamine-Sensitizing Doses (HSD_{50}) for HSFS Mice[a]

Strain	Generations 11–14[a] $\dfrac{HSD_{50}}{HSD_{50}}$	Generations 18–20 $\dfrac{HSD_{50}}{HSD_{50}}$
$\dfrac{\text{N:NIH(SW) } ♀}{\text{HSFS/N } ♀}$	$\dfrac{3.29}{0.51}=6.45$	$\dfrac{2.01}{0.50}=4.02$
$\dfrac{\text{N:NIH(SW) } ♂}{\text{HSFS/N } ♂}$	$\dfrac{3.34}{0.64}=5.21$	$\dfrac{3.37}{0.70}=4.81$

[a] Ref. 3.

Pertussis Vaccine Potency Test

Male and female mice from generations 11–14 of HSFS/N and HSFR/N strains and females of N:NIH(SW) were immunized with pertussis vaccine lot 7b (3). There was a period of 14 days between vaccination and challenge with *B. pertussis*. A separate virulence control was used for each strain and sex. The same was done with male and female mice from generations 18–20 of HSFS/N and N:NIH(SW). The vaccine-median-effective dose (ED_{50}) and virulence-control (LD_{50}) estimates for each strain and sex were calculated by Wilson-Worcester analysis.

Table 5 summarizes ED_{50}s and LD_{50}s for the HSFS/N and HSFR/N strains after generations 11–14. The comparative ratios are given in Table 6. The ED_{50} and LD_{50} are indications of the immunizability and susceptibility to challenge of each of the strains. Comparisons of the HSFR/N mice with HSFS/N mice show that it requires 8.05 times more vaccine to immunize a resistant female than a sensitive female, and it requires 6.25 times more vaccine

for resistant males than for susceptible males. Comparisons of the LD_{50}s for the HSFR/N and HSFS/N strains indicate that 2.26 and 3.02 times more challenge-culture is required respectively for sensitive females and males than for resistant females and males. Thus, in selecting for increased immunizability as indicated by the reduced ED_{50} in the HSFS/N strain, there has also been a decrease in susceptibility to challenge as indicated by the LD_{50}. Likewise, the HSFR/N strain is less immunizable but has an increased susceptibility to challenge. Compared to the N:NIH(SW) females, the HSFR/N females required 4.90 times more vaccine for immunization but were 2.16 times more susceptible to challenge. The HSFS/N females required only 0.61 of the vaccine needed to immunize the N:NIH(SW) females and had essentially the same susceptibility to challenge.

The ED_{50}s and LD_{50}s for the HSFS/N strain after 18–20 generations of selection are given in Table 7, and the comparative ratios are given in Table 8. The results of the ED_{50} assays from generations 11–14 indicate that the HSFS/S strain is more easily immunized than the N:NIH(SW) parent stock. In contrast to these data, the results from generations 18–20 indicate that the HSFS/N strain is not more easily immunized than the N:NIH(SW). The ratios of the ED_{50}s indicate that both female and male HSFS/N mice require approximately the same amount of vaccine for immunization as the parent

Table 5. Summary of Pertussis Vaccine Potency and Virulence Control Tests[a] for Generations 11–14

Strain	Sex	ED_{50} (ml)	1 S.D. (%)	Number of Mice	LD_{50} Dil	1 S.D. (%)	Number of Mice
HSFS/N	♀	0.00774	82–121	192	430	79–127	206
HSFS/N	♂	0.00621	87–115	190	245	83–120	207
HSFR/N	♀	0.06231	79–126	191	972	83–120	208
HSFR/N	♂	0.03886	86–116	189	739	88–114	196
N:NIH(SW)	♀	0.01260	96–105	928	451	88–106	544

[a] Ref. 3.

Table 6. Ratios of Immunizability and Susceptibility to Challenge[a] for Generations 11–14

Strain	$\dfrac{ED_{50}}{LD_{50}}$	$\dfrac{LD_{50}}{ED_{50}}$
$\dfrac{\text{HSFR/N } ♂}{\text{HSFS/N } ♂}$	$\dfrac{0.03886}{0.00621}=6.26^c$	$\dfrac{739}{245}=3.02^c$
$\dfrac{\text{HSFR/N } ♀}{\text{HSFS/N } ♀}$	$\dfrac{0.06232}{0.00774}=8.05^c$	$\dfrac{972}{430}=2.26^b$
$\dfrac{\text{HSFS/N } ♀}{\text{N:NIH(SW) } ♀}$	$\dfrac{0.00774}{0.01260}=0.61^b$	$\dfrac{430}{451}=0.95$
$\dfrac{\text{HSFR/N } ♀}{\text{N:NIH(SW) } ♀}$	$\dfrac{0.06232}{0.01260}=4.90^c$	$\dfrac{972}{451}=2.16^c$

[a] Ref. 3.
[b] p<0.05
[c] p<0.001

Table 7. Summary of Pertussis Vaccine Potency and Virulence Control Tests for Generations 18–20

Strain	Sex	ED_{50} (ml)	1 S.D. (%)	Number of Mice	LD_{50} Dil	1 S.D. (%)	Number of Mice
HSFS/N	♀	0.0167	81–123	1228	247.2	83–120	1228
HSFS/N	♂	0.0164	81–123	804	265.2	89–112	1093
N:NIH(SW)	♀	0.0123	89–113	1125	345.7	88–113	968

Table 8. Ratios of ED_{50} for HSFS/N Mice

Strain	Generations 11–14[a] $\dfrac{ED_{50}}{ED_{50}}$	Generations 18–20 $\dfrac{ED_{50}}{ED_{50}}$
$\dfrac{\text{HSFS/N } ♀}{\text{N:NIH(SW) } ♀}$	$\dfrac{0.00774}{0.01260}=0.61$	$\dfrac{0.0167}{0.0123}=1.35$
$\dfrac{\text{HSFS/N } ♂}{\text{N:NIH(SW) } ♀}$	$\dfrac{0.00621}{0.01260}=0.49^b$	$\dfrac{0.0164}{0.0123}=1.33$

[a] Ref. 3.
[b] p<0.05

N:NIH(SW) mice. The reason for the regression of the immunizability of the HSFS/N strain from generations 11–14 to generations 18–20 is not known. The most recent measurements of the $ED_{50}s$ from generations 18–20 for the HSFS/N strain do not agree with the data reported by Manclark et al. (3) and Pittman (4), who found a positive correlation between histamine sensitivity and immunizability. Future assays of the $ED_{50}s$ for the HSFR/N strain after generations 18–20 may help resolve this apparent discrepancy.

Genetic Profiles

Isozymes are enzymes with different molecular forms that can be distinguished by their physical and chemical properties. Genetic differences have been found in a large variety of isozymes from many of the commonly used inbred strains (5).

The HSFS/N and HSFR/N strains have been assayed for 12 biochemical workers using the procedures referred to by Roderick et al. (5). The genetic profiles for the HSFS/N and HSFR/N strains for these 12 loci are given in Table 9. Both strains were homozygous for all loci tested, as would be expected after 20 generations of sib-matings. The two strains carry the same alleles at all loci except for hemoglobin beta-chain (Hbb) and malic enzyme (Mod–1). HSFS/N carries the *d*-allele of Hbb and the *a*-allele for Mod–1; HSFR/N carries the *s*- and *b*-alleles for Hbb and Mod–1 respectively.

Discussion

Two strains of mice, HSFS/N(susceptible) and HSFR/N(resistant), have been developed by selectivity breeding for their ability to be sensitized to histamine by pertussis vaccine. The responses after 20 generations of selection indicate that susceptibility to sensitization to histamine is a heritable trait and thus can be changed through selective breeding. The HSFS/N and HSFR/N strains have been shown to differ widely in their susceptibility to the lethal effects of histamine as measured by the median histamine-sensitizing dose. The mice from the HSFS/N strain are easily sensitized to the lethal effects of histamine and easily immunized with pertussis vaccine.

Assays for 12 biochemical markers in the HSFS/N and HSFR/N strains indicate that they are isogenic lines. Once a genetic profile is established it is possible to distinguish one strain from all others. The ability to differentiate strains could be of considerable importance in the future. If these strains become widely used in bioassays, it will be necessary

Table 9. Genetic Profiles for 12 Loci

Locus	Chromosome	HSFS/N	HSFR/N
Major urinary protein	4	a	a
Hemoglobin beta-chain	1	d	s[a]
Transferrin	9	b	b
Isocitrate dehydrogenase	13	a	a
Dipeptidase	13	b	b
Glucose-6-phosphate dehydrogenase	8	b	b
Phosphoglucomutase	17	a	a
Glucosephosphate isomerase	1	b	b
Esterase-1	18	b	b
Esterase-3	7	c	c
Malic enzyme	2	a	b[a]
Catalase	17	a	a

[a] Loci for which the two strains have different alleles

to establish colonies in many different locations. Since genetic contamination is a constant threat in an animal facility that houses more than one strain of a particular species, a genetic profile can be used to constantly monitor the genetic integrity of an animal colony.

REFERENCES

1. Hansen, C. T.; Manclark, C. R.; Pittman, M.; Hinkle, W. J. Selective breeding for HSF sensitivity in the mouse. Spiegel, A., ed. The laboratory animal in drug testing. Fifth International Committee on Laboratory Animal Symposium, Hannover, 1972. Stuttgart: Gustav Fischer Verlag; 1973:151–156.
2. Hinkle, W. J. A comparison of lifetime reproductive performance of inbred mice and non-inbred mice on two feeding regimes. College Park, Md.: University of Maryland; 1971. Thesis.
3. Manclark, C. R.; Hansen, C. T.; Treadwell, P. E.; Pittman, M. Selective breeding to establish a standard mouse for pertussis vaccine bioassay: II. Bioresponses of mice susceptible and resistant to sensitization by pertussis vaccine HSF. J. Biol. Stand. 3:353–363; 1975.
4. Pittman, M. Mouse strain variation in response to pertussis vaccine and tetanus toxoids. International Symposium on Laboratory Animals, London, vol. 5, 1966. Symposia Series in Immunological Standardization. Basel, New York: Karger; 1967:161–166.
5. Roderick, T. H.; Ruddle, F. H.; Chapman, V. M.; Shows, T. B. Biochemical polymorphisms in feral and inbred mice (*Mus musculus*). Biochem. Genet. 5:457–466; 1971.

International Standards for Bacterial Content of Vaccines

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ABSTRACT

Dry weight and nitrogen determinations are suitable for standardization of bacterial content of vaccines. Data presented show that they have several advantages over an opacity tube as an international standard: 1) they are definable; 2) they ignore bacterial size and shape; 3) they are not altered by heating at 56° for 30 minutes, dialysis, or storage at 30°C in the presence of thimerosal and therefore permit reevaluation or confirmation of bacterial content during the processing of vaccines; and 4) they can serve as a basis for calibration of photometric instruments and opacity reference tubes. Data were accumulated from samples taken during routine production. Vaccines made in our laboratories have demonstrated satisfactory potency, toxicity, and safety as required by the U.S. Code of Federal Regulations. These vaccines, diluted to 18 OU/ml, contain mean values of 0.540 mg dry wt/ml, 0.06 mg trichloracetic acid-precipitated nitrogen/ml, and 0.077 mg total nitrogen/ml. The values are consistent and reproducible.

INTRODUCTION

The World Health Organization (WHO) specifies three methods for determining the bacterial content of vaccines: 1) opacity, for pertussis, typhoid, and cholera vaccine; 2) nitrogen content, for typhoid and cholera vaccines; and 3) dry weight, for BCG (1). Of these methods, opacity determinations are the most expedient and economical. The WHO provides an International Opacity Reference Preparation (IORP) assigned an opacity of 10 International Units per ml (2). The first IORP suspension was prepared by WHO using the suspension of ground glass that had been used in the preparation of the first U.S. Opacity Standard (USOS); and although in 1953 both preparations were equally opaque visually and photometrically (3, 4), comparison of the third IORP and the fifth USOS showed differences of 40 to 84% (5,6).

Spaun (7) noted a decrease in density of the IORP due to glass particles adhering to the inner wall of the tube. The problem of instability of the ground glass was circumvented by adopting a plastic rod for IORP #5 (1,8,9). This rod simulates the optical properties of bacterial suspensions.

The WHO and the United States recommend different methods for comparing the density of cell suspensions. The WHO recommends visual comparison (3,10–12); whereas the United States requires photometric readings (4,13).

Differences in opacity readings in different photometric instruments became apparent with the sec-ond IORP. Spaun (14) recommended that only bacterial suspensions with the same extinction coefficient be compared. Furthermore, due to differences in optical design, the opacity values differed from instrument to instrument. He therefore concluded that the opacity of suspensions of each organism should be specified for each instrument in terms of the IORP.

Results between laboratories differ for several reasons: the two different references; different methods of reading (visual or photometric); differences between instruments; and differences in the time of sampling for opacity determinations. The WHO and the United States require that the opacity of a pertussis suspension be determined within 2 weeks of harvesting and prior to any chemical or physical process capable of altering the opacity (15,16). This justifiable requirement nevertheless prevents the reevaluation or confirmation of bacterial content by opacity during processing of the vaccine; thus it is not permissible to correct a wrong initial measurement or to reassess a bacterial concentration after mishaps affecting concentration.

Twenty-five years of international efforts to establish opacity as a means of measuring the bacterial content of pertussis vaccine have not resulted in a method that permits comparison between laboratories.

The dry weight and nitrogen methods, already recognized by the WHO for determination of the bacterial content of other vaccines, have decided

advantages when compared with opacity. These methods 1) can be precisely defined, 2) can serve as a basis for calibration of photometric instruments and reference tubes, 3) are unaffected by bacterial size and shape, 4) are suitable for use in intermediate stages of vaccine production, 5) are applicable to the standardization of extracted and purified vaccines, and 6) are readily performed in most laboratories.

METHODS

Licensed Vaccines

Strains of *Bordetella pertussis* isolated from patients with whooping cough were freeze-dried. Prior to vaccine production, cultures were reconstituted by plating on Bordet-Gengou (BG) agar (17) and were maintained with biweekly transfers. Cultures were seeded into bottles of BG medium and incubated at 35°C for 72 hours (Table 1). The bacteria were washed from the medium with phosphate-buffered saline (PBS), pH 6.9, and the suspension was centrifuged. The supernatant was discarded, and the sediment was resuspended in PBS and held for 10 days at 3°C until completion of purity tests. Opacity was measured in the pooled harvests, before thimerosal was added. Single-strain concentrates were combined and diluted with PBS on the basis of the opacity determination made prior to the addition of thimerosal. Formalin is sometimes added to detoxify vaccines.

Developmental Vaccines

A 24-hour culture, maintained on BG agar as above, was scraped into Stainer-Scholte (SS) medium (18) in Erlenmeyer flasks and incubated for 24 hours on a reciprocal shaker. A second subculture in liquid medium was used to seed 1.5 l of SS medium, which was incubated at 34°C with air flowing over the surface and with stirring at about 500 rpm. After 24–28 hours incubation, the culture was used to seed 14 l SS medium in a New Brunswick fermentor. These fourth subcultures on liquid media were incubated at 34°C with stirring at 300 rpm and air blown over the surface. After 40–45 hours the bacteria were concentrated by removing the culture medium through a Selas filter (19). The bacteria were collected and diluted in PBS to a concentration of about 250–300 OU/ml. The opacity was determined before heating at 56–58°C for 30 minutes and adding thimerosal.

Bacterial Mass

Duplicate samples of vaccines containing at least 10 mg dry weight for each of four samples were dialyzed against running, distilled water for 24 hours. Samples were shown to be free of chloride as determined by $AgNO_3$. Dialyzed and nondialyzed vaccines were weighed in open, tared, aluminum pans and held at 60–65°C for 24 hours. The pans were weighed, returned to the oven, and reweighed until the weight was stable within 0.2 mg. The

Table 1. Outline of Pertussis Vaccine Production and Sampling for Opacity Determination

	Michigan Department of Public Health		WHO-Copenhagen
	Licensed	Developmental	
Medium	Bordet-Gengou and peptone	Stainer Scholte liquid medium (Tris only to adjust pH to 7)	Bordet-Gengou "modified"
Incubation	70 hr @ 35° C	42 hr @ 34° C	48 hr @ 37° C
Removal of culture	Washed	Filtered (Selas)	Manually scraped off
Suspending fluid	Phosphate-buffered saline, pH 6.9; centrifuged and resuspended	PBS, pH 6.9; diluted to ca. 300 OU/ml OPACITY	Saline + 0.04% formalin; homogenized, ca. 80 OU/ml
Time and temp held	10-14 days @ 4° C OPACITY	56° C for 30 min	5 days @ 22–25° C centrifuged
Preservative	0.02% Thimerosal	0.01% Thimerosal	Saline + 0.01% thimerosal
OU/ml	500–700	250–300	OPACITY to 160 OU/ml
Approximate vol, single strains	1 l	3 l	1 l
Combined strains	100 or 125 OU/ml (calculated), 0.01% thimerosal		OPACITY adjusted to 160

standard deviation of the Christian Becker analytical balance is 0.04 mg as determined by the method of Olesen Larsen, Lyng, and Spaun (20).

Opacity

All results are expressed as U.S. Opacity Units (OU). A Coleman Junior II spectrophotometer set at 530 nm was adjusted to 50% transmission (T) with the USOS. Measured volumes of vaccine were diluted with measured volumes of saline until the spectrophotometer read 50% T. From these dilution ratios, the opacities were calculated.

Nitrogen Determinations

Microassays of nitrogen were made by the method of Ma and Zuazaga (21). Protein nitrogen was precipitated in 5% trichloracetic acid (TCA–N), allowed to stand overnight, centrifuged, and washed three times with 5% TCA.

RESULTS

In estimations of bacterial mass by determination of dry weight, Spaun (22,23) and Csizer et al. (24) weighed samples of vaccines and subtracted the weight of salt. Three lots of autoclaved PBS had values of 11.1 mg/ml, 11.4 mg/ml, and 12.0 mg/ml, an excess of 0.7–1.6 mg/ml over the calculated weight of 10.4 mg/ml. The percent weight of salt from PBS varies with the concentration of bacteria. At 700 OU/ml the salt constitutes about 30% of the total weight—at 100 OU/ml, almost 78%. Parent lots containing 100 OU/ml with only 3 mg of bacterial weight per ml showed artificially high values when the calculated weight of salt was subtracted (Portwood, unpublished data). Samples with low bacterial mass were therefore dialyzed. Table 2 lists the number of vaccines, the standard deviation, and the 95% confidence intervals of dry weight before and after dialysis. Bacterial mass is designated as μg/OU of harvest in order to make values

comparable. There is no significant difference in the bacterial mass of vaccines at the 95% level of confidence by the Student t-test. Other factors thought capable of affecting bacterial weight were examined: 1) medium; and 2) heating at 56°C for 30 minutes. There was no significant difference between the dry weight of bacteria cultivated in liquid SS medium and grown on solid BG medium or between those heated at 56° C and those not heated.

There were differences in the dry weights of different strains of B. pertussis adjusted to the same opacity, as shown in Table 3. The mean μg/OU of 37 vaccines representing 203 replicates was 29.9 with a standard deviation of 2.2. There is a significant difference between the bacterial mass of strains at the 95% level of confidence. This is expected since the size and shape of particles alter opacity. The strain differences provide additional evidence of the advantage of dry weight as a way of measuring bacterial content.

Table 4 shows the bacterial mass of parent lots of vaccine composed of three strains of B. pertussis

Table 2. Bacterial Mass of Pertussis Vaccines Under Various Conditions

| Condition | μg/OU | | | | | |
	Mean	n	s	95% limits	t_0	t/0.95
Dialysis						
before	30.0	19	2.0	29.0 to 30.9	0.8	2.1
after	29.2	18	2.4	28.0 to 30.4	−0.7	2.1
Medium						
Bordet-Gengou	30.0	14	1.7	29.0 to 31.0	0.4	2.1
Stainer-Scholte	29.4	23	2.4	28.3 to 30.4	−0.5	2.1
56° C for 30 min						
not heated	29.8	21	1.8	28.9 to 30.6	0.8	2.1
heated	29.3	16	2.7	27.8 to 31.5	−0.6	2.1

OU: opacity units
n: number of replicates
s: standard deviation

Table 3. Bacterial Mass of Strains of Bordetella pertussis

Strain Number	μg/OU Mean	n	s	95% Limits	t_0	t/0.95	Significance
10536	29.3	9	1.7	28.0 to 30.6	−0.3	2.3	average
18336	28.5	17	2.1	27.5 to 29.6	−1.8	2.1	90%
18904	31.3	11	1.9	30.1 to 32.6	2.6	2.2	95%
All strains	29.6	37	2.2	28.9 to 30.3			

OU: opacity units
n: number of replicates
s: standard deviation

Table 4. Bacterial Mass and Opacity After Periods of Storage

Lot No.	OU-h/ml	Storage (months)	Opacity OU-s/ml	Mass (mg/ml)	μg/OU-h
516	125	2.5	106	3.17	25.4
		3.5	100	3.48	27.8
		45	89	3.72	29.8
523	100	1	82	3.03	30.3
523 F 0.025% formalin		7	n.d.	2.78	27.8
		8	n.d.	2.77	27.7
		36	73	3.09	30.9
531	100	1	n.d.	3.04	30.4
		17	66	3.03	30.3
532	100	3.5	n.d.	3.20	32.0
		24	70	2.99	29.9
533	100	1	80	3.15	31.5
		7	76	3.08	30.8

OU-h: opacity units at harvest
OU-s: opacity units after storage
n.d.: not done

and diluted to 100 or 125 OU/ml. They were standardized within 2 weeks of harvesting and prior to the addition of preservative, then later diluted and stored at 3°C for up to 45 months. The mean μg/OU of 12 vaccines was 29.9 with a standard deviation of 1.5 and 95% confidence intervals of 26.6 to 33.2. With one exception the dry weights of the parent lots after storage were within the 95% confidence intervals.

The opacity and mass of vaccines produced at the Michigan Department of Public Health (MDPH) were compared with those produced at Statens Seruminstitut (WHO–C) (22). Differences between laboratories in sampling are given in Table 1. Opacity determinations at MDPH were made on freshly harvested live suspensions of bacteria, in contrast to WHO–C vaccines, which had been formalinized, homogenized, held at room temperature for 5 days, centrifuged, and the sedimented bacteria resuspended in saline containing thimerosal before determination of opacity. The opacity of the final MDPH bulk vaccines was determined at time of harvest, while WHO–C vaccines were adjusted to a given opacity unitage. The MDPH vaccines standardized with USOS have 30 μg/OU. Vaccines produced at Statens Seruminstitut, standardized by WHO reference and containing 16 OU/ml have a mass of 0.3220 mg/ml, or 20 μg/OU. Therefore the bacterial mass of vaccines from WHO–C at a given opacity is one-third less than MDPH vaccines at the same opacity.

Nitrogen

Parent lots of vaccine containing three strains of *B. pertussis* were assayed for total nitrogen (TN) and trichloracetic acid precipitated nitrogen (TCA–N) by micro-Kjeldahl. Both assays were included to check for consistency. Media were removed from all vaccines either by centrifugation or by filtration prior to standardization by opacity and estimation of nitrogen. Table 5 includes data from parent lots and from adsorbed pertussis vaccines. No statistically significant difference was found between nitrogen values for parent lot and adsorbed vaccines. Assays of 20 lots gave values of 3.38 μg TCA–N/OU with a standard deviation of 0.3. The mean value for total nitrogen assays was 4.31 μg/ml with a deviation of 0.4. There was a statistically significant ratio between the TCA–N and TN values for parent lots and adsorbed vaccines, an average of 0.79 with an estimated standard deviation of 0.07. The determinations of TCA–N and TN are sufficiently reproducible and consistent to recommend both assays.

DISCUSSION

Determination of opacity is the most rapid, convenient, and economical method for measuring bacterial content in culture suspensions. The method is satisfactory when assays are done in a designated laboratory, with standardized instruments and reference preparations, and on bacteria that have not been subject to physical or chemical treatments that can alter opacity. It is the method of choice for preparing cultures for challenge in the mouse protection test, for determining the concentration of bacteria in vaccines prior to dilution, and for comparing yields of various production methods. However, it is misleading when comparing results be-

Table 5. Nitrogen Values of Parent Lots (3 strains) of Pertussis Vaccines and Pertussis Vaccines-Adsorbed

	μg TCA-N/OU			μg TN/OU			TCA-N/TN		
	mean	n	s	mean	n	s	mean	n	s
Parent lots	3.46	10	0.30	4.49	6	0.15			
Pertussis vaccine— adsorbed	3.30	10	0.31	4.21	10	0.46			
Total	3.38	20	0.3	4.31	16	0.40	0.79	16	0.07

TCA-N: trichloracetic acid precipitated nitrogen
TN: total nitrogen
OU: opacity units
n: number of vaccines
s: standard deviation

tween laboratories. The existence of two different opacity reference preparations, the instability of the ground glass reference preparations, and the different readings given by different photometers for the same opacity reference all make it impossible to compare results between laboratories. Measurement of opacity *per se* as a reference is undesirable. Opacity must be calibrated and defined by some absolute method such as estimation of bacterial mass or bacterial nitrogen.

Two satisfactory assays for measuring bacterial content are dry weight and nitrogen. We concur with Spaun (7) that dry weight determinations provide a suitable and reproducible method for controlling the bacterial content of vaccines. Based on opacity at the time of harvest, there were no significant differences in the bacterial mass of cells grown on BG or SS medium. There were changes in opacity but not in bacterial mass after dialysis, heating at 56° C for 30 minutes, or storage at 3° C. There were differences in the bacterial content of vaccine from different strains diluted to the same opacity.

Our data demonstrate the suitability of nitrogen content as an indicator of bacterial content. The total nitrogen and trichloracetic acid precipitable nitrogen content of concentrates and of finished bulk vaccine were consistent and the assays were reproducible. Nitrogen content is already used as a standard for some bacterial vaccines. For typhoid vaccine, WHO regulations permit the measurement of either nitrogen or opacity (25). U.S. regulations specify a maximum acceptable nitrogen content in typhoid (26) and cholera (27) vaccines and require specific nitrogen values for extracts of typhoid vaccine and TCA–N for cholera vaccine. Nitrogen assays require less time and use appreciably less product than dry weight assays. They can also be made on small amounts of the final plain or adsorbed products, whereas dry weight assays need much more material.

The most important properties of bacterial vaccines are potency and safety. There are three requirements for typhoid vaccine: potency, opacity, and total nitrogen content. For interlaboratory comparison of vaccine quality, it is essential to have a way of assigning an absolute value to the biologically active components.

The data we have presented were accumulated from samples taken during routine production of vaccines. The samples were assayed by standard methods and showed satisfactory potency in the mouse potency test. The efficacy of these vaccines can be inferred from the decreased incidence of whooping cough in the community during the last 40 years. Vaccines diluted to about 18 OU/ml contain 0.540 mg dry weight/ml, 0.06 mg TCA–N/ml, and 0.077 mg TN/ml. These values should be verified by other laboratories making vaccines that meet WHO requirements of potency and safety.

Knowledge of nitrogen content and of dry weight would make comparison of vaccines between laboratories more meaningful, and this in turn would lead to the development of improved products.

SUMMARY

An international standard for measuring the bacterial content of pertussis vaccine and for use in interlaboratory comparisons should be an absolute standard unaffected by bacterial size and shape and appropriate for standardizing extracts, purified preparations, and whole cells.

The confusion over the different WHO and U.S. opacity reference preparations, changes in the preparations with age, different readings in different photometers, and unsuitability for use with purified vaccines make opacity reference preparations unsatisfactory as standards.

Two parameters suitable for the bacterial content of vaccines are dry weight and nitrogen. Nitrogen values can be used in all stages of manufacture and estimates of dry weight can be used for standardizing photometric instruments. Vaccines prepared at the Michigan Department of Public Health at a concentration of 18 OU/ml average 0.06 mg trichloracetic acid-precipitated nitrogen/ml, 0.077 mg total nitrogen/ml, and 0.540 mg bacterial mass/ml. These methods, unlike measurements of opacity, are not influenced by changes in the vaccine during physical or chemical processes.

Knowledge of nitrogen content and of dry weight would make comparisons between laboratories preparing vaccines to WHO specifications more meaningful. It would also permit exchange of information between laboratories, which could lead to the development of improved products.

ACKNOWLEDGMENTS

REFERENCES

1. W.H.O./I.A.B.S. symposium on the measurement of the number of organisms in bacterial vaccines. J. Biol. Stand. 4:69–70; 1976.
2. W.H.O. Tech. Rep. Ser. 68:7–8; 1953.
3. Maaløe, O. The international reference preparation for opacity. Bull. W.H.O. 12:769–775; 1955.
4. Pittman, M. History, benefits and limitations of Pyrex glass particle opacity references. J. Biol. Stand. 4:115–125; 1976.
5. Geser, G. Problems arising when determining the number of bacteria in a pertussis suspension. International symposium on pertussis, Bilthoven, 1969, Symp. Series Immunol. Stand. 13:36–38; 1970.
6. Joó, I. Some principles and problems of pertussis vaccine preparation. International Symposium on Pertussis, Bilthoven, 1969, Symp. Series Immunobiol. Stand. 13:49–50; 1970.
7. Spaun, J. Studies on reproduction of the international opacity reference preparation. Bull. W.H.O. 26:213–217; 1962.
8. Perkins, F. T.; Sheffield, F. W.; Outschoorn, A. S.; Hemsley, D. A. An international collaborative study on the measurement of the opacity of bacterial suspensions. J. Biol. Stand. 1:1–10; 1973.
9. W.H.O. Tech. Rep. Ser. 594:16–17; 1976.
10. W.H.O. Tech. Rep. Ser. 86:14; 1954.
11. W.H.O. Tech. Rep. Ser. 259:26; 1963
12. W.H.O. Tech. Rep. Ser. 329:21; 1966.
13. Seligmann, E. B., Jr.; Farber, J. F. The use of the U.S. opacity standard. J. Biol. Stand. 4:127–130; 1976.
14. Spaun, J. Problems in standardization of turbidity determinations on bacterial suspensions. Bull. W.H.O. 26:219–225; 1962.
15. W.H.O. Tech. Rep. Ser. 274:33; 1964.
16. Code of Federal Regulations, Part 620.2 (b), 620.11 (c) (1), 620.31 (c) (1); 1977.
17. Diagnostic procedures and reagents. New York: American Public Health Association, Inc.; 1963: 134.
18. Stainer, D. W.; Scholte, M. J. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. J. Gen. Microbiol. 63:211–220; 1970.
19. Cohen, Arthur L. Rapid and simple methods for the harvesting of mass cultures of microorganisms. Appl. Microbiol. 1:168–171; 1953.
20. Olesen Larsen, S.; Lyng, J.; Spaun, J. Estimation of the precision of a balance. Dansk. Tidsskr. Farm. 42:107–116; 1968.
21. Ma, T. S.; Zuazaga, G. Micro-Kjeldahl determination of nitrogen. Ind. End. Chem. 14:280–282; 1942.
22. Unpublished working document W.H.O./B.S./72.1061.
23. W.H.O. Tech. Rep. Ser. 530:9; 1973.
24. Csizér, Z.; Szammet, A.; Niedermayer, N.; Zsidai, J.; Auber, E.; Joó, I. Estimation of bacterial mass of pertussis vaccine by opacity and dry weight determinations. J. Biol. Stand. 5:289–295; 1977.
25. W.H.O. Tech. Rep. Ser. 361:69–70; 1967.
26. Code of Federal Regulations. 620.11 (d); 1977.
27. ibid. 620.31 (d).

DISCUSSION OF PART 4

DR. PITTMAN: I would like to offer a hypothesis which I think may be the key to the pathogenesis and immunity of whooping cough.

Induction of a bacterial disease is dependent on the specific antigen that causes the harmful effects of the disease. Immunity is dependent on the antibody to a specific antigen(s). Running through the literature are threads which indicate that a specific toxin liberated by *Bordetella pertussis*, localized and multiplying among the cilia of the respiratory tract, causes the harmful effects of whooping cough and prolonged immunity.

What is the nature of this toxic substance (Table 1)? It is not the 56° labile toxin. This toxin is present also in *Bordetella bronchiseptica*, yet the clinical symptoms of this infection in animals are different from those of pertussis. *B. bronchiseptica* does not have lymphocytic and histamine-sensitizing properties. Immunity after the infection does not last for more than a few months.

The specific toxic substance of *B. pertussis* has the characteristics of a protein exotoxin as defined by van Heyningen. Like other exotoxinoses (diphtheria, tetanus, botulism, cholera), pertussis is a receptor cell disease. The activity of the toxin is mediated by messages from the receptor cell(s). For example, an active extract of *B. pertussis* has no direct effect on the release of insulin from B-islet pancreatic cells in in vitro culture. Islet cells from mice and rats 3 days after intraperitoneal injection of the pertussis extract, treated with the extract, secrete a significantly increased amount of insulin.

The nature of pertussis immunity is shown in Table 2. Many years ago Standfast, Dolby, and associates pointed up two protective antibodies. One protected mice against the intracerebral challenge and the other against the lethal intranasal challenge. Intracerebral protection appears to be antihesive in addition to possessing other antibacterial activities. Protection against lethal intranasal or intraperitoneal challenge is antitoxic. There is little or no proliferation of the bacteria. Death is due to toxemia and can be prevented with convalescent whooping cough serum which has no anti–HLT reactivity.

The front line of defense is to prevent the bacteria from adhering to the cilia. This protection is short-lived. Persons intimately exposed to whooping cough may have positive respiratory tract cultures. Yet if they have had whooping cough or been recently vaccinated, typical clinical symptoms of whooping cough usually do not develop. Antitoxin, the second line of defense, acts against the 80°-labile toxin (HSF–LPF), not against the 56°-labile toxin.

If my interpretation is correct, pertussis vaccine should be capable of inducing not only antiadhesive antibodies, but also antibodies against the 80°-labile toxin. This new approach would surely lead to methods for toxoiding the specific reactive factor in pertussis vaccine and to the development of in vitro methods for the evaluation of immunity.

DR. MALONEY: I have two comments on the position paper of Dr. Cameron. He referred, among many other topics, to a paper by Finney and associates, who were investigating alternative approaches to the statistical analysis of results of potency tests. According to their paper, they found that it would not be possible to pool slopes. Almost a year ago, I analyzed accumulated data from the Bureau of Biologics and, while I have to go entirely by memory, I believe we found somewhat different results from those reported by Finney et al. The conclusions are that if data are analyzed from one source, they may be a very accurate record of what happens in that circumstance and not an accurate picture of what happens elsewhere.

I went on to investigate what gain in efficiency would be possible under altered testing circumstances and altered statistical analysis. I remind you that Dr. Cameron referred to the enormous mouse and workload involved in potency testing. Under ideal circumstances, according to me anyway, it is possible to attain a gain of up to tenfold. Remember that is under ideal circumstances, of course. But it would seem to me it would be possible relatively easily to make a material gain at any rate.

Table 1. Pertussis Toxin Has the Characteristics of a True Toxin

1. It causes the harmful effects of whooping cough.
2. It is neutralized by homologous antibody.
3. It is less stable to heat than endotoxin.
4. After fixation to receptor cell, antibody is of no apparent value.
5. It has its own pharmacology. Mediation is through the adrenergic autonomic neuron system.

Table 2. Whooping Cough: A Cell Receptor Disease

Disease	*B. pertussis* adheres to cilia, multiples and liberates toxin(s) Toxin attaches to receptor cell(s) Reactions—pharmacological, metabolic, neurological, etc.

Prevention	Front line
	    Prevention of adhesion of bacteria
	Second line
	    Toxin neutralization or receptor cell blockade

Protective Antibody	Anti-adhesive and other anti-bacterial effects
	    Protects against intracerebral challenge
	Antitoxin
	    Protects against lethal intranasal and lethal intraperitoneal challenge

DR. SPIERS: I would like to ask Dr. Cameron to explain what he meant by hypersensitivity tests. Are they related to what we now call reaginic antibody and that type of hypersensitivity, or did he have something else in mind?

DR. CAMERON: The hypersensitivity I was talking about stems from work I had been doing with *Escherichia coli* many years ago. Essentially we are talking about the Shwartzman phenomenon and the fact that mice injected with *E. coli* lipopolysaccharides can be exquisitely sensitive for a period of 24 to 48 hours, and beyond that period lose the sensitivity completely.

I have been trying to offer an explanation for the sporadic nature of reactions in pertussis. You cannot conceivably, I think, lay these wholly at the door of the vaccine itself. I think we are talking about a two-component system, the child and the vaccine, and I am simply trying to hypothesize, to give us something to work on.

DR. SPIERS: Maybe, to follow that up just a little bit, this is a different type of sensitivity. We do know that pertussis vaccine can act as an adjuvant for IgE production if it is given with tetanus or diphtheria toxoids. I will have more to say about that later. The presence of IgE antibody can lead to many local reactions and occasional anaphylactic reactions. We are now getting to a point where we are much better able to measure IgE antibody. There are now radioimmunoassays for human IgE, and we are developing similar methods in the mouse.

DR. CAMERON: In a sense we are talking about almost the opposite insofar as what we have to hypothesize, if you like, is the means of primary sensitization. Pertussis is not the agent involved here. The question we are asking ourselves is: how does the child acquire such sensitization that when it is injected with pertussis vaccine, it reacts so violently? What is the causative agent at this point? This is why I am suggesting the possibility of what I call heterologous sensitization with lipopolysaccharides from Gram-negative organisms other than *B. pertussis*, which we know are at that time in the intestine.

DR. MANCLARK: There are many of us who question the value of the mouse weight gain test. Does Dr. Csizer have any evidence that the preparations that he used at different toxicities in the mouse weight gain test have been correlated in any way with clinical evidence of toxicity? Would you also indicate what you think of the reproducibility of the mouse weight gain test?

DR. CSIZER: About the reproducibility, we evaluated the homogeneity of the data according to Finney, and according to this, the test was valid, which means that in our hands this test is reproducible.

DR. MANCLARK: And how about the relationship between these toxic preparations in the mouse compared to what happens in the clinic?

DR. CSIZER: We have data in Hungary on every routine batch of DTP vaccine collected by the National Vaccine Control Laboratory at the National Institute of Health in Hungary. For each routine DTP batch, which is about 500,000 doses, at least 50 infants are monitored after three injections for 6 months, both as to the immunogenicity and toxicity of the vaccines. But as far as

I know, there is no relationship between the data presented here and the clinical data, which means that our vaccine is potent and the reactivity is moderate.

DR. HENNESSEN: Dr. Csizer, you said your vaccines contain 10 opacity units, if I remember correctly. Could you tell us what this would mean in international units of potency? A second question is: in your slides, the regression lines were so fantastically parallel, both for the adsorbed and the nonadsorbed material. Could you explain this? Would you not expect that they would be nonparallel for the adsorbed and for the unadsorbed material? And a third question is: are these lines, which are very close together, really significantly different for the different preparations?

DR. CSIZER: In answer to the first question, for the nonadsorbed preparation, the mean protective value or potency in the last 10 years has been about 18 per 30 international opacity units, and about 12 to 13 in the case of absorbed DTP vaccine.

DR. PERKINS: That is very high.

DR. CSIZER: In answer to your second question, those lines are very nicely parallel because of the statistical handling and not because of the experimental data. That was why I showed the first series of slides, in which I presented or tried to present the actual weight gain data. You could see some lines crossing but not if you handled the data as a whole, which cover 19,584 observations. It is the sheer quantity of data which makes these lines parallel.

With the exception of the first day body weight difference, they were parallel. For all the others, all the lines were statistically straight and parallel. My paper submitted for publication contains the whole series of statistical evaluations and there you can see the point better.

DR. J. B. ROBBINS: I do not want to make a comment about this paper directly, but to focus attention on the problem of testing pertussis and combined vaccines in general. I echo your sentiments that it is very likely that with a little bit of luck and perhaps some additional money, we will have a better vaccine in the future, but we still have the problem of dealing with the vaccine today. I think that the subject should not be recorded as hopeless, even though the bioassays are so complex. For instance, the effects measured in the mouse weight gain test are mediated in part by the LPS. Studies could be conducted to compare the mouse weight gain test with a single component of the LPS, such as the KDO sugar. Perhaps a chemical assay, which relies upon a chemical standard rather than a bioassay, could be studied by several laboratories to see if this could be used as an alternative to the mouse weight gain test.

As Dr. Pittman indicated, the intracerebral challenge is really a function of adhesion or more specifically a function of adhesion to the ciliated cells or the ventricles of the brain, and it might be that a more direct measure of adhesion or of inhibition of adhesion could be conducted with systems such as that mentioned by the group from North Carolina.

The control testing of pertussis vaccine and pertussis-containing products requires a tremendous amount of time and effort by both the control laboratories and manu-

facturers. I think it would be worthwhile to try to combine some new chemistry and physics with our bioassays to see if we can more accurately and simply measure what the bioassays attempt to measure.

DR. PERKINS: If you had accepted those methods when we devised them, I would be very happy. The ball is right at your feet, and you know it.

DR. RELYVELD: Should we not speak of adjuvanted instead of adsorbed vaccine? In many cases, adsorption is more or less complete.

Let me take as an example, aluminum hydroxide and aluminum phosphate. When using aluminum phosphate with tetanus and diphtheria toxoids, you can have 20–40% of the antigen free in the supernatant, whereas with aluminum hydroxide, you can have 100% adsorption. The same is true for *B. pertussis* cells. With aluminum phosphate one can see under the microscope that many of the organisms are not adsorbed.

But when you use aluminum hydroxide you will find that, depending on the quality of the preparation, you may have complete adsorption or only a small fraction of the bacteria nonadsorbed.

Now to come back to what Dr. Cameron said, when I titrated firmly adsorbed pertussis vaccine, I found as he did that before adsorption you can have high protective titers, whereas after adsorption, the protective titer falls. Therefore, it is not true that the potency of the vaccine is diminished, but only that the immunogenicity, determined in mice, is altered by adsorption.

DR. STAINER: I would agree with Dr. Relyveld. I also looked at so-called adsorbed vaccines, and you do not see adsorption even under the electron microscope. You see a whole range of cells surrounded by particles of aluminum phosphate gel. So strictly, we should not talk about DTP adsorbed. On another subject I would like to report an observation made when we were developing the synthetic medium. The medium gave us higher opacities, and the culture was detoxified with the same concentration of formalin as the routinely prepared vaccine. We assayed the culture and found that it did not pass the mouse weight gain test. This was understandable, I suppose, because there were more organisms. So we added more formalin and retested. It passed the mouse weight gain test and then we centrifuged the vaccine and stored it as a pilot lot. A month later, the vaccine appeared as a stringy, mucilaginous mixture. We knew that the amount of formalin that we had added had detoxified the vaccine, but what we were also concerned about was how to stabilize it so that it would not transform into the mucilaginous material. We therefore titrated the extra formalin necessary to achieve this. When this was done, the concentrated vaccine remained as a stable, smooth suspension with good antigenic potency. The Danish workers use 0.04% formalin for 5 days at room temperature. If I did that to my vaccine, I could guarantee that if I didn't use it within 2 weeks, I would have problems. I think if you use a vaccine that has this potential for stickiness and add adjuvant to it, you may be looking at something totally different. You could be looking at something which potentially could be toxic.

DR. CAMERON: I should like to come back to what Dr. Relyveld said and suggest that we be careful not to sidetrack ourselves on this question of adjuvant. He said that in many cases vaccines with aluminum added to them are not adsorbed, and he suggested the use of the term "adjuvated." I suggest that many of them are not "adjuvated" either, but that is another matter. Let us consider some other vaccines, pasteurella and erysipelothrix, for example. These vaccines come into the same category. Aluminum phosphate or aluminum hydroxide is added and, whether we argue as to whether or not they are adsorbed or "adjuvated," there is no doubt that in both cases there is a considerable enhancement of the antigenic response. This is what concerns me. Regardless of the name, regardless of the use of the English language, does adjuvant/adsorbent boost the antigenic response? I do not believe it does. I think there are enough people here today who have said this quietly in the past and I should now like to repeat the question: does adjuvant confer any benefit in terms of an improved immune response in the child? It certainly does not seem to do so in the mouse.

DR. MANCLARK: I would like to react, not so much respond, to Dr. Stainer's comment of a few moments ago. I do not really feel I should say too much because I shall be referring to the work of people who are not here. The group at North Carolina that is studying the physiology and genetics of pertussis has some good evidence that if you have a mucilaginous suspension, as described, the breakdown of the bacteria is not a function of the method of detoxification. You are probably dealing with a degraded strain that should not be used in vaccine production.

DR. CHRISTENSEN: I think I agree with Dr. Relyveld. Our experiences with aluminum hydroxide in DTP vaccine, and the clinical data I mentioned yesterday, were based upon immunization with DTP vaccine containing aluminum hydroxide during the 60s; the vaccine contained on average 10 mouse protective units/ml, as against the vaccine used since 1970, which is nonadsorbed and with the same number of organisms contains only about 4 units/ml. They correlate fairly well with the clinical data, so we believe in the adsorption if you mean adsorption, and you only get adsorption with aluminum hydroxide, as Dr. Relyveld said.

Dr. Blaskett, I wish to ask you one question. In which volume did you inject your material—0.5 or 0.2 ml?

DR. BLASKETT: I do not think the volume is important. It is the amount of thimerosal.

DR. CHRISTENSEN: It is. That is why I ask.

DR. BLASKETT: The amount of thimerosal is fairly important, but how we got onto this was that when I was in the United States about 6 years ago—I think it was in Michigan—I was told that they had found evidence that a particular number of organisms gave a less toxic result in the mouse weight gain test if injected in a larger volume.

When I returned to Australia, we talked about this, and we decided that we would investigate it ourselves. Unfortunately, we included some thimerosal in the diluent, the reason being that we knew that Gotschall et al. in Michigan had discovered that thimerosal had

an effect on toxicity in the weight gain test. We thought we would not like to change the concentration of thimerosal, without realizing that the factor we should have been dealing with was the quantity of thimerosal.

We found that our intussusceptions occurred particularly when we injected half of a single human dose in 0.5 ml of thimerosal saline, whereas if we injected in 0.5 ml of ordinary saline or even 0.25 ml of undiluted vaccine, we did not tend to get them.

DR. CHRISTENSEN: What I said was that when we noticed this phenomenon in 1971, it was because we had taken over the production of pertussis vaccine, and we continued to test according to the WHO recommendations, i.e., an injection of 0.5 ml. Then we had a number of deaths among the mice that the veterinarian described as caused by intestinal invagination. We wondered what was happening. On looking back in the protocols we discovered that earlier vaccines did not give such deaths. But the test done on the old material had been injected in 0.2 ml. We found that it made a difference if the 8 opacity units was injected in 0.5 ml or in 0.2 ml. We tried to learn, using dyes, whether toxicity could be related to the area of spread after injection. That was our impression, but it was indeed very difficult to substantiate. But volume does make a difference.

DR. BLASKETT: All I can say is that we have had intussusceptions with 0.25 ml volumes with the correct amount of pertussis and thimerosal present, and we have also had intussusceptions with both 0.5 ml and 1.0 ml volumes. In our hands it has depended on the amount of thimerosal, but I agree that volume also plays a role.

DR. PEETZ: I have three technical questions for Dr. Hertz. How are the cells fractionated in the antigen preparation, was there any detergent in the agarose, and what was the electroendoosmotic coefficient of the agarose?

DR. HERTZ: The antigenic preparation was a 48,000 g supernatant of sonicated organisms without detergent. We have used detergents for other purposes, but the slides shown referred to antigen without detergent. The endoosmotic coefficient $(-M_r)$ was -0.15.

DR. SPEIRS: I would like to ask Dr. Smith and possibly Dr. Manclark, as they selected this strain of mouse for susceptibility or resistance to histamine sensitization, what effect did that have on other responses to pertussis, such as the adjuvant effect or the LPS and so on? Were any tests done along that line?

DR. MANCLARK: We have not completely characterized these mice yet. In our 1975 publication, we gave some characteristics of the mice. For example, the HSFR/N mice are quite susceptible to endotoxin. But we have not done anything else with any of the other pertussis components. I think Dr. Hewlett mentioned earlier that we are using the HSFS/N mouse for the insulin work, and it is a remarkably useful animal for such studies. The characteristic we are interested in here is not the fact that the mouse responds one way or another to some agent; it is our goal to reduce the variability in the response to selected agents.

DR. MUNOZ: You mentioned that you have had some problems, but you did not say what problems, and I would like very much to know.

DR. K. P. SMITH: In generation 22 we had a striking decrease in the HSD_{50}s, and in the susceptibility values that we have been measuring in each generation. At the time it happened, we thought it was some unknown environmental problem and that the mice had contracted a low grade infection that would probably resolve itself. However, in generation 23 we noticed a similar problem. The susceptible strain was much less easily sensitized. There were two explanations: environmental contamination and genetic contamination.

These are albino mice and we have many albino strains maintained in the same facility. Genetic monitoring in generations 21 and 22 did not indicate an overt outcross. Some drug treatments—dichlorvos and piperazine—were also suspected and are still under review. Preliminary data suggest that this is probably not involved.

Another possibility is the testing procedure. Even though it is a simple test procedure, there is always the possibility of error.

DR. MUNOZ: During my work on *B. pertussis* I have run across across two occasions in which most of the colony of mice that we were using was destroyed. We used the mice that survived to start another colony. The original colony was easily sensitized to histamine. Within a very short period of time, the new colony was almost totally inadequate for the histamine test. This happened to us at Merck, Sharp and Dohme as well as in Montana. I understand that Carworth Farms has had similar problems. I do not know if the present colony can be sensitized by pertussis vaccine, but Dr. Wright told me that the mice he was purchasing from Carworth Farms were no longer susceptible to sensitization. These variations were not clearly genetic.

DR. MANCLARK: What you have said is what caused us to start this whole study. We wanted to have a mouse that had predictable responses and we wanted to have a mouse that we could monitor before a test was done. I think we are close to having that mouse now, and it will also be useful for some other things.

I think that Dr. Smith can respond to the concerns you have about barrier maintenance and so forth, but we have been through this problem.

DR. K. P. SMITH: I feel fairly confident for another reason that this is not a genetic change, "either a mutation" or an overt outcross. This is indicated from results in generation 23. I have noted in the procedures that we test two different litters usually from the first two parities. From one mating pair in generation 23, we have seen sensitivity up to 70 percent and for the second litter of this same pair the susceptibility value was zero percent, thus there is no correlation between the two litters. This indicates to me that this is an environmental problem, either in the animal facility or in the testing facility, or in the testing procedure.

These mice were caesarean-derived into a barrier facility at generations 13 or 14, and we saw no difference in the response to histamine at that time. As was seen from the data I showed today, the response continued upward after caesarean derivation.

DR. MUNOZ: I just want to say that we are going to be disappointed in thinking that we can have a uniform

mouse. When these mice are taken to different laboratories in the world they will again react differently even if the genetic makeup is identical.

DR. K. P. SMITH: I have no doubt that you are right about that; there will be differences shown as you take these animals from one laboratory to another. But within any one laboratory we can hope to have a predictable response, something we cannot count on at present.

DR. PERKINS: I think that that experiment was done by Dr. Pittman 20 years ago when she supplied everyone with a group of the NIH mice that had been bred for years at the NIH but as many different colonies were grown out as there were laboratories breeding them.

DR. NOVOTNY: You may be interested in an observation which arose during the testing of a *Corynebacterium parvum* preparation for histamine sensitizing activity. We have two strains of mice called "NIH." They certainly do not look like your mice, but the grandparents were from NIH. One of these lines is easily sensitized by *Corynebacterium parvum*, but not with *Bordetella pertussis*. The reverse is true for the other line. I wonder whether it would be helpful in tracing your markers to use both *Bordetella pertussis* and *Corynebacterium parvum* as a histamine sensitizing agent, because it does appear that they differ according to the mice used.

DR. MANCLARK: Dr. Novotny sent us some *C. parvum* a few weeks ago. We have not had a chance to try it yet but we will certainly do that soon.

MISS JAKUS: I would like to mention the frustration I have in testing pertussis vaccine. The ED_{50} of pertussis vaccine varies with the strain of mice and with the immunizing dose range.

Figure 1 suggests a linear relationship between optical density and relative potency for reference vaccines from 5 sources tested last year. Given that the International Standard is 8 immunogenic units/ml, the other references gave potencies: U.S. #7 = 9.9; #4 = 12.4; #66/
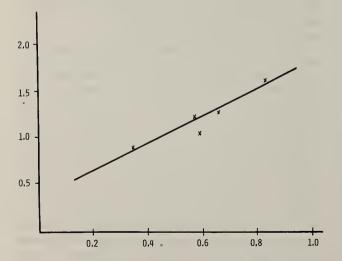
$302 = 8.1$; #66/303 = 9.5; #66/84 = 7.0 immunogenic units/ml, as calculated from Table 3.

DR. CANADJIJA: I have just one comment regarding the influence of mouse strains which we used in the potency control of pertussis vaccine. According to our experience there is a difference in immunological response not only between two different strains of mice, but also between two lines of the same strains. We tested the national reference pertussis vaccine against the international standard in 36 mouse protection tests over a period of 10 years. For testing, we used mice received from NIH in 1965 marked as NIH BXS. After a period of fairly constant results from 1967 to 1970, we noted that the ED_{50} values started to increase gradually but steadily for both the international and national standard. These observations forced us to undertake an analysis of several factors which may influence the mouse protection test.

We found that almost in parallel with the increase of ED_{50} values, the condition of the mouse colony deteriorated. This was manifested by an increased number of deaths between immunization and challenge. Starting with 1974, we introduced a new line of the same strain of mice which we received from the NIH in 1973. This strain was identified as N:NIH (SW). At the end of 1974, we began to use mice derived from the new line, and ED_{50} values showed a clear downward trend for both the international and national standard preparations. It is worth notice that mice which we received in 1965 were from conventional breeding stock and the line of mice received in 1973 were from a specific pathogen free colony. We supposed that such differences in ED_{50} values may be caused by different conditions for breeding and holding the mice.

DR. K. P. SMITH: To comment upon that, there are several things about the NIH Swiss-Webster stock. First of all, at the present time the mating scheme is a randomized circular pair mating scheme, which is a true random scheme devised to minimize the amount of inbreeding over time. I am not sure when this scheme was introduced, but I would guess around 1965 or 1966. Prior to that time, there was something called a random brother-sister scheme. If I had a nightmare as a geneticist, that is the worst possible mating scheme I could think of for any use whatsoever in animal breeding. When someone told me this was a scheme used at

Figure 1. Plot of average estimates of five pertussis standards relative to the International Standard for Pertussis (BB method) versus optical density.

Table 3. Estimates of Average Potencies of Five Pertussis Standards Relative to the International Standard for Pertussis

| Preparation | Optical Density at 8 I.U. per ml | Evaluated by U.S. BB Criteria | | |
		Relative Potency	95 Percent Confidence Limits	Weights
66/302	0.599	1.01	(0.67, 1.51)	122
66/303	0.587	1.19	(0.80, 1.78)	128
U.S. #4	0.840	1.55	(0.77, 3.15)	41
U.S. #7	0.660	1.24	(0.69, 2.25)	58
66/84	0.350	0.88	(0.54, 1.44)	86

one time prior to 1965, prior to when Carl Hansen started managing these colonies, that is the most bastardly system I have ever heard of. It is no wonder results were so unsatisfactory under such a management scheme.

The present scheme is one of the best schemes possible that I know of in managing a random mating colony, which is not an easy job at all to do correctly. This strain has also been in a specific pathogen-free environment for the last 10 years, which could also have a great effect upon the immunological tests that are performed on these animals.

DR. MANCLARK: When that change was made to the SPF environment, the mice were exhaustively tested and there was no difference between the two environments.

DR. COX: I am a commercial breeder of mice and rats and have to agree that one of the main secrets of success is following Dr. Hansen's randomization chart. If you have a minimum of 50 pairs or more, then there should be no trouble. If Dr. Hansen's scheme is not followed, you will get into inbreeding and you will cause a drift in the genetic background of your mice.

DR. WARDLAW: This is not a question to the last speaker of the session, but it is a comment from a confused user of opacity standards. My understanding is that a fundamental change in terminology has been introduced in going from the ground glass standard to the plastic rod, and I think this should be commented on because if the matter is not resolved, we will just have perpetuation of an unnecessary source of difficulty. My understanding is that if you are using the ground glass suspension and you wish to talk about bacterial concentration, you describe your data in terms of *opacity units per ml* because the label on the ground glass standard states "10 opacity units per milliliter." That means that if you want to have some measure of bacterial mass or numbers, without getting into the argument of the equivalence between opacity units and milligrams or billions, you refer to total bacterial mass or total bacterial numbers as having an equivalent of so many *opacity units*. I think this is the sense which most of the speakers at this symposium have used. My understanding is that when the plastic rod was issued, this terminology was thrown out and if we now wish to talk about bacterial concentration, we describe it in terms of *opacity units* without any "per milliliter," because the plastic rod is labeled as 10 opacity units. This new opacity unit has built into it the concept of "per milliliter," so that it becomes meaningless with the plastic rod to use the term *"opacity unit per milliliter"* because that would physically have the dimensions of mass divided by volume squared, which has no correspondence in physical reality. This means that if one wishes to talk about bacterial mass or numbers—for example, if you want to describe how much pertussis has been injected into a mouse—then the correct term, logically, is the term which caused Dr. Perkins such concern, which is the *"opacity unit milliliters."* That is, it is the number of opacity units multiplied by the number of milliliters, and is the expression of total bacterial dose. So that if, for example, you have a bottle of pertussis vaccine that contains a liter of 10 opacity

unit material, the total amount of bacteria in that bottle is 100,000 *opacity unit milliliters*. You can spin that down and resuspend it in whatever volume you want. You are still dealing with 100,000 *opacity unit millliters* of pertussis. If you resuspend it back to a liter, of course, you have 10 opacity unit material if you wish to talk about concentration. But I would like, as a confused user, to put it to the meeting that we should, if we accept the plastic rod, be using the term *opacity unit* when we are describing *concentration,* and we should be using the term *opacity unit milliliter,* not per milliliter, but *opacity unit milliliters* if we are talking about total bacterial mass, total numbers, or total dose.

DR. MANCLARK: I should like to congratulate Dr. Portwood on her presentation. Everything she has said is quite valid and I support it. One of our problems is that, as organizations and individuals, we have some ego involvement with some of the standards, and one of the first things, I think, that we should do is to remove the Opacity Standard as a standard. It is a useful reference material, but that is all it is. The only place where it has any real value is in the standardization of bacterial suspensions for challenge, but you can use any method that will give you a proper plate count. I think the methods that Dr. Portwood has indicated are important. One of the most important things is that you can check a vaccine, a final vaccine, to see if it contains what the manufacturer says is contained in the vaccine.

DR. STAINER: I would agree with both of the last speakers in that we do need some clarification. Recently we did some similar work to Dr. Portwood's. We determined dry weights, nitrogen, and DNA levels. This was reported at a previous meeting. The person who does the determinations is a very careful worker, everything had been done in triplicate, and the results were very good with very little scatter. He was in great consternation when he compared our results to those published by Spaun. Our results were out by a factor of 1.8 compared with Spaun's, because, of course, we had used the U.S. opacity standard.

DR. PERKINS: We produced a plastic rod to stop people from using the international opacity reference preparation incorrectly, which is to put it in a nephelometer; that should never be done. We made a plastic rod therefore to stop scientists from doing this, because they will go on doing it if we supply a suspension. The reference has to be compared by naked eye. That is the only way of comparing two turbid preparations, because the nephelometer—whatever instrument used—is dependent upon the physical shape, the size of the particle, and how many particles are suspended in a particular suspension medium. The eye is the only instrument that will correct for the variables, and direct observation is the only way in which you can measure opacity.

DR. CAMERON: We are skirting the heart of this problem and not really getting at the problem. At the moment, I believe all of us know that, for practical purposes, vaccines in North America contain almost twice as many organisms as vaccines in Europe. Until both groups begin using the plastic rod, or using anything

you like, provided both use the same standard this very serious discrepancy will continue. I think this is what we should be talking about stopping.

DR. PERKINS: If you all wish to have the international opacity unit withdrawn and to quote the concentration of your pertussis vaccines in protein nitrogen, please write to the WHO and tell us. But we all must use the same standard. There *are* two standards of opacity in this world and this is the only instance in which there is a national standard in addition to an international standard. It is obligatory for the member countries of the United Nations to use International Standards where they exist, and the International Opacity Standard is not being used in the United States.

DR. CHRISTENSEN: I just want to remind you that I think we have dropped any opacity standard of reference in the European Pharmacopoeia. We are only giving values for the potency.

DR. WARDLAW: I am sorry to belabor this, but I was wondering if you would care to offer comments on the use of opacity unit milliliters in the terms I suggested.

DR. PERKINS: I have no comment.

Part 5. PERTUSSIS VACCINE EXPERIENCE AND OTHER APPROACHES FOR THE CONTROL OF PERTUSSIS

Chairman: *Hans Cohen*
Rapporteur: *J. Nagel*

Vaccination Against Pertussis, Yes or No?

H. Cohen

This is an important session, because in some countries workers interested in the problems of immunization against communicable diseases are confronted, perhaps for the first time in their careers, with the effect of negative publicity by mass communication on public acceptance of immunization programs, including pertussis vaccination. One has to be a stranger in Jerusalem not to realize that public acceptance of an immunization procedure determines its success or failure.

One of the things public health authorities can do when faced with a situation like this is carefully analyze the available scientific data and confront scientists in favor of or against immunization with the epidemiological facts.

I assume there is a consensus here on the validity of the results of the controlled field trials in the United States and Great Britain 20 years ago. Are these results still valid? Do today's improved social conditions, including better housing, less crowding, better food, and better medical care, reduce the risk of serious complications or death from pertussis, even though they do not completely prevent outbreaks of the disease? Recent analysis of the pertussis epidemic in the United Kingdom, to be presented in this session, might yield sufficient data to answer this question, at least partially.

A second question deals with the properties of the pertussis vaccine. In the past, great attention has been given to the relation between animal potency tests and effectiveness of the vaccine for man. Less attention has been given to the safety of the vaccine for man. No one today can deny that at least some pertussis vaccines cause unwanted side effects, in some cases serious ones. Data on how often irreparable neurologic sequelae occur are scarce. The occurrence of complications may depend on the method of vaccine production.

Some countries like Denmark and the Netherlands have answered the question "Pertussis vaccination, yes or no?" with a cautious, "Yes, but . . .", and have reduced the concentration of the pertussis component in the vaccine. Some data on the effect of this reduction are now available. Measures like this, however, should be framed by a set of contraindications to be evaluated by physicians before they advise parents to have their infants vaccinated against pertussis. A service should be established to be contacted during the postvaccination period by concerned parents.

Qualified pediatricians should be available to visit vaccinees at home on short notice when this is judged necessary. These physicians should also be available to instruct the personnel of baby clinics and the general public on the benefits and risks of pertussis immunization. It is also very desirable that specially trained medical doctors be engaged in the quality control of vaccines.

Although several biologically active components of *B. pertussis* have been identified, we still do not know much about their pharmacologic activities in babies or how important those activities are.

The papers in this session may well contribute to our understanding of some of these questions.

Pertussis Vaccine in the United States: The Benefit-Risk Ratio

E. A. Mortimer, Jr., and P. K. Jones

ABSTRACT

Mortality from pertussis in the United States has declined remarkably since 1900. Attribution of this decline to pertussis vaccine has been difficult because crude mortality rates from pertussis declined 82% between 1900–1904 and 1935–1939, prior to the widespread use of pertussis vaccine beginning in the 1940s.

Because part of this apparent decline might be a consequence of shifts in the age distribution of the population during this century, we calculated age-specific mortality rates from pertussis for infants less than 1 year old and for children 1 to 4 years old for the years 1900–1974, using published vital statistics.

The results show a decline for both age groups both before and after advent of the vaccine, but the slopes after appearance of the vaccine were steeper. Examination of the mortality slopes (calculated by least squares) showed a significantly steeper fall for infants ($p < 0.01$) but not for older children for the years 1940–1954 compared to 1930–1939.

The maximum and average declines between consecutive 5-year periods in the years 1900–1939 were calculated and extrapolated through 1974 for both age groups. Comparison of deaths expected to those observed for 1940–1974 shows 87–90% fewer deaths than expected in the last 35 years. During 1970–1974, fewer than 1% of the deaths expected by extrapolation from 1900–1939 were observed. Assuming that these differences are attributable to pertussis vaccine, examination of rates of serious reactions ascribed to the vaccine suggests the vaccine's benefits far outweigh the risks.

No preventive or therapeutic measure, including pertussis vaccine, is absolutely safe or absolutely effective. For every such measure some estimate of a benefit-risk ratio must be made, and when there are alternative measures comparisons must be made. It is our purpose to examine alternative approaches to the control of pertussis and in particular to assess the effect of widespread use of pertussis vaccine on mortality from the disease in the United States.

In the United States vaccination is generally considered to be the optimum way to minimize morbidity and mortality from pertussis. Alternatives to immunization include control of transmission by such means as quarantine, administration of hyperimmune human serum globulin or erythromycin to contacts and early cases, treatment of complications of the disease, or some combination of these.

Studies of the efficacy of these alternatives to immunization, though few, indicate limited usefulness. Unfortunately, the disease is highly contagious in the catarrhal stage prior to the development of recognizable stigmata. Moreover, older children and adults whose immunity—acquired naturally or by immunization—may have faded, may experience mild pertussis with atypical manifestations and thereby become unidentified transmitters. (Whether asymptomatic carriers of *B. pertussis* exist is doubtful.) Nonetheless, it is possible that some of the decline in mortality from pertussis that occurred prior to immunization resulted from mandatory or voluntary quarantine and consequent limiting of exposure of children until school age, when case fatality rates are minimal (1). Nevertheless, it seems clear that prevention of exposure is an unreliable way to ensure maximum control of pertussis.

How effective are current measures for preventing or modifying the disease in exposed, susceptible individuals? Studies of antimicrobial agents have indicated some benefits. Erythromycin appears to be the most effective and is associated with little or no risk or side effects (2). Co-trimoxazole may also have merit (3). But exposure must be recognized, and often it is not. Once the symptoms of the

disease have begun, beneficial effects from erythromycin decline. By the end of the catarrhal stage of the disease (2 weeks after onset of symptoms and less than 3 weeks after exposure), no effect may be expected (4).

Studies of human hyperimmune and convalescent sera prior to widespread use of the vaccine suggested that these sera frequently prevented or ameliorated the disease in exposed, susceptible infants (5). Such sera carry the risk of hepatitis B. More recent fractionated preparations of human sera (Pertussis Immune Serum Globulin, Human) do not, but there is no evidence that this new preparation has any prophylactic or therapeutic merit (6). This suggests that effective serum antibody to pertussis, if any exists, is carried in the IgM rather than the IgG fraction of human γ-globulins. Thus passive immunization as a control measure for pertussis appears useless.

How effective are current therapeutic measures in patients with established pertussis? Although erythromycin may reduce the duration of carriage of the organism, no effect on symptoms has been demonstrated (4). Immune serum globulin has no value (7). No measure has been shown to prevent or ameliorate pertussis encephalopathy once the disease is established. Furthermore, postmortem studies of infants and children succumbing to pertussis in the 1930s indicate that few died from complications, such as pneumococcal pneumonia, that would be susceptible to therapies developed since that time (8).

These prophylactic and therapeutic alternatives to widespread pertussis immunization seem to have minimal risk, but unfortunately, the benefit side of the benefit-risk ratio is unacceptably low on the basis of current evidence.

The accepted approach to control of pertussis in the United States and much of the world is therefore pertussis vaccine. But pertussis vaccine is associated with some risks, and doubts have been expressed about its efficacy (the benefits).

Risks of Pertussis Vaccine

Few physicians doubt that pertussis vaccine occasionally produces severe reactions with permanent sequelae or death. Unfortunately, the frequency of these severe reactions is not precisely known. Moreover, pertussis vaccine produces some peculiar milder reactions that are disturbing but of unknown importance.

Minor transient reactions include fever of varying degree; irritability; and redness, swelling, and pain at the site of injection. Unless extreme, such as very high fever, these frequent minor reactions are generally considered inconsequential. Moreover, since pertussis vaccine is usually given with an aluminum salt adjuvant and in combination with diphtheria and tetanus toxoids (DTP, adsorbed), it may be difficult to determine the precise contribution of the pertussis component.

There are three disturbing reactions to pertussis vaccine or perhaps to one or more components of the combined vaccine that are of unknown or uncertain importance. The first is excessive somnolence, beginning within a few hours of the injection. The second is an episode of one to several hours of high-pitched, persistent, uncontrollable screaming, beginning within a few hours of the injection. The high-pitched quality of the cry, reminiscent of an "encephalitic cry," is particularly worrisome to pediatricians. The third and rarest is an episode of one to several hours of shock with all its manifestations. Death from this rare but frightening reaction has not been recorded, and its pathogenesis has not been determined.

The occurrence of occasional central nervous system reactions to pertussis vaccine is well established. These occur almost always within 48 hours after injection and range from a simple short-lived convulsion to intractable convulsions, encephalopathy with permanent brain damage and, rarely, death.

Precise estimates of the quantitative and qualitative importance of these reactions cannot be made. Review of the considerable literature related to the toxicity of pertussis vaccine uncovers multiple reasons why such estimates cannot be developed (9-51). Many early studies provided no denominators. Equally important, in some studies denominators are only estimated, and in some instances it is unclear whether the determined rates of reactions relate to numbers of children or numbers or injections. Most of the studies are retrospective. There are few controlled studies that permit estimates of background rates of symptoms that might be attributed erroneously to pertussis vaccine. Many studies are confounded by the use of combined vaccines, whose other components may be reactive. Differences in vaccines may also account for differences in rates of reactivity. There is a lack of standardized definitions of reactions. What is irritability? What is excessive screaming? What is somnolence? What is a simple convulsion? What is encephalopathy?

Finally, the importance of some of the reactions, especially somnolence and excessive screaming, is unknown. Do they represent mild forms of encephalopathy that produce permanent cerebral damage that is ignored as simple variation in inherent intellectual ability? These, and perhaps other factors, prevent estimation of the risks from pertussis vaccine with any confidence.

Benefits of Pertussis Vaccine

Partly because of concerns about the toxicity of pertussis vaccine, its efficacy has been scrutinized in recent years. For two reasons doubts have been raised as to how effective it is. First, current pertussis vaccines have had few controlled field trials of their ability to prevent disease. The original studies of pertussis vaccines in the thirties were poorly designed and were performed with unstandardized vaccines of uncertain and variable potency (52). However, several more recent field trials have shown an effect (28,53). Second, mortality from pertussis declined considerably in this country and elsewhere in the first 40 years of this century, before the widespread use of vaccine (Table 1). This has suggested to some that the recent negligible mortality from pertussis in the United States and the United Kingdom may simply represent a continuation of this secular trend and other factors, rather than being an effect of the vaccines (13,54).

Possible explanations for this downward trend prior to the vaccine are multiple and difficult to assess. They may well have contributed to the continuing decline in mortality after the development of standardized vaccines. Among these factors are differences in the nature of population denominators as the death registration areas of the United States were expanded from 1900 to 1933; differences in diagnostic criteria; the decreased birth rate during the Great Depression in the thirties; the effects

of antibiotics on some complications; possible but undeterminable changes in the organism that altered its virulence; the effects of enhanced diagnosis and voluntary or involuntary quarantine; and other factors such as socioeconomic status and nutrition, which cannot be evaluated.

One contributing factor that may be assessed is that of the changing age distribution of the population. Since the rates shown in Table 1 are crude and not adjusted for the increasing longevity of the population from 1900 to the present, and because most of the mortality from pertussis occurs in infants and young children, a decline in crude mortality rates might be misleading. More than 95% of mortality from pertussis in unimmunized populations occurs before the fifth birthday. In 1900, 12.1% of the population was under 5 years of age, in contrast to 8.1% in 1940. The proportions of the population under 5 in 1960 and 1970 were 11.3% and 8.4% respectively.

To adjust for these disparities in the age distribution of the population, we calculated age-specific mortality rates from pertussis for infants less than 1 year old and for children 1 to 4 years old in 5-year periods from 1900 to 1974. The number of deaths from pertussis was obtained from published vital statistics. Populations at risk were identified by the National Center for Health Statistics for the years 1900 to 1940 and by published vital statistics for subsequent years. Table 2 shows average annual death rates in 5-year periods for these two age groups, and Figure 1 represents 5-year moving aver-

Table 1. Average Annual Crude Mortality Rates for Pertussis in 5-Year Periods, United States, 1900–1974[a]

Years	Deaths/100,000	Years	Deaths/100,000
1900–04	17.4	1940–44	2.1
1905–09	14.3	1945–49	0.7
1910–14	11.5	1950–54	0.4
1915–19	10.8	1955–59	0.2
1920–24	7.2	1960–64	0.1
1925–29	6.9	1965–69	<0.1
1930–34	4.6	1970–74	<0.1
1935–39	3.1		

[a] For death registration areas only prior to 1933

Table 2. Average Annual Age-Specific Mortality Rates for Pertussis in 5-Year Periods, United States, 1900–1974[a]

Years	Children Less than 1 Year Deaths/1000	% Decrement	Children 1–4 Years Deaths/1000	% Decrement
1900–04	4.34		0.87	
1905–09	3.65	16	0.69	21
1910–14	2.96	19	0.55	21
1915–19	2.60	12	0.50	7
1920–24	2.31	11	0.39	23
1925–29	2.05	11	0.33	15
1930–34	1.66	19	0.23	30
1935–39	1.30	22	0.16	33
1940–44	0.86	34	0.09	44
1945–49	0.34	60	0.03	66
1950–54	0.12	64	0.01	90
1955–59	0.05	62	<0.01	62
1960–64	0.02	64	<0.01	70
1965–69	0.01	53	<0.01	30
1970–74	<0.01	63	<0.01	71

[a] For death registration areas only prior to 1933
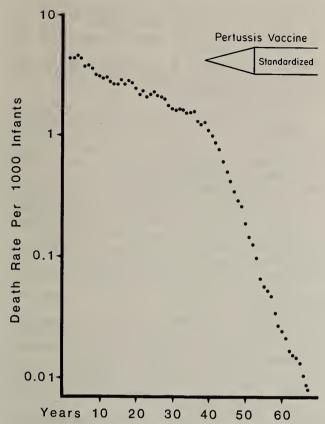
Figure 1. Pertussis deaths per 1000 infants aged less than one year. Five-year moving averages, 1902–1971.

age death rates between 1902 and 1971. It is again clear that a distinct decline in mortality from pertussis occurred long before the development and widespread use of pertussis vaccines. (It is not possible to specify a precise year or 5-year period during which pertussis vaccines became widely used. Experimental pertussis vaccines were developed in the early thirties and by the mid-forties were widely used. However, the vaccines were not standardized by government regulation until 1947. Current potency requirements were developed in 1953.)

Examination of mortality slopes, calculated by least squares, for successive five-year periods shows a significantly steeper decline for infants less than 1 year old (p <0.01) but not for children 1 to 4 years old during 1940–1954 compared to 1930–1939.

Although projections are tenuous, it is interesting to predict the expected recent mortality from pertussis if the trends of 1900 to 1939 had persisted. In that period the average 5-year decrements in mortality for infants less than 1 year old and in children 1 to 4 years old were 16 and 21% respectively (Table 2). Persistence of those decrements would have resulted in 108,000 deaths from per-

tussis in children under 5 between 1940 and 1974 (35 years), in contrast to the 10,620 observed. Even in 1970–1974, 8,362 deaths would have been expected; 52 were observed. Extrapolation of the maximum 5-year decrements of 22 and 33% in these two age groups would still have resulted in 78,000 deaths in 1940–1974, and 4,409 in 1970–1974.

It is not possible to attribute this salutary trend to pertussis vaccine with any degree of confidence. It is not due to a lowered birth rate, because the birth rate increased after World War II. The effects of improved social and economic conditions are not possible to determine. The accelerated decline in mortality did coincide with the development of increasingly effective antimicrobial therapy, but autopsy studies of children succumbing to pertussis show that the major causes of death are peribronchial pneumonia, atelectasis, other pulmonary lesions, and encephalopathy, none of which is likely to be ameliorated by antimicrobial therapy. Few die of pneumococcal lobar pneumonia or other more manageable lesions (8).

The Benefit-Risk Ratio

Assuming that the lower-than-expected mortality from pertussis in the United States between 1970 and 1974 is indeed due to vaccine, can we determine a benefit-risk ratio for the vaccine? Here we are hampered by the lack of precise data on the rates and importance of vaccine reactions. Moreover, death is not the only significant complication of pertussis itself; encephalopathy occurs more commonly after pertussis than after the vaccine, although precise rates are not known.

Stewart has estimated that rates of encephalopathy following pertussis vaccine are as high as 2 per 100,000 doses, a figure others say is too high (55). From 1970 to 1974, 16,850,000 children were born in the United States. If these children averaged four doses of DTP each at the rates estimated by Stewart, 1,348 cases of encephalopathy should have occurred (270 annually), a figure that seems somewhat higher than anecdotal experience would indicate. But extrapolation of mortality trends (see above) suggested that more than 4,000 deaths from pertussis would have occurred during these 5 years (800 annually) in the absence of pertussis vaccine. The number of pertussis encephalopathy cases with survival and subsequent intellectual damage that would have occurred cannot be estimated.

Our data are admittedly estimates and extrapolations that do not consider all the vagaries of biol-

ogy. Nonetheless, even accepting the highest estimates of encephalopathy due to pertussis vaccine and the most modest estimates of benefits from the vaccine, there is clearly more benefit than risk from the vaccine on the basis of the experience in the United States.

REFERENCES

1. Mortimer, E. A., Jr. Immunization against infectious disease. Science 200:902–907; 1978.
2. Altemeier, W. A., III; Ayoub, E. M. Erythromycin prophylaxis for pertussis. Pediatrics 59:623–625; 1977.
3. Cullen, A. S.; Cullen, H. B. Whooping-cough: prophylaxis with co-trimoxazole. Lancet 1:556; 1978.
4. Baraff, L. J.; Wilkins, J.; Wehrle, P. F. The role of antibiotics, immunizations and adenoviruses in pertussis. Pediatrics 61:224–230; 1978.
5. Lapin, J. H. Whooping cough. Springfield, Ill. Charles C Thomas; 1943:175–182.
6. Morris, D.; McDonald, J. C. Failure of hyperimmune globulin to prevent whooping cough. Arch. Dis. Child. 32:236–239; 1957.
7. Ames, R. G.; Cohen, S. M.; Fischer, A. E.; et al. Comparison of the therapeutic efficacy of four agents in pertussis. Pediatrics 11:323–336; 1953.
8. Lapin, J. H. Whooping cough. Springfield, Ill., Charles C Thomas; 1943:136–145.
9. Madsen, T. Vaccination against whooping cough. J.A.M.A. 101:187–188; 1933.
10. Wilson, G. Hazards of immunisation. Lancet 2:456–457; 1967.
11. Anonymous. Vaccination against whooping cough. Brit. Med. J. 3:539–540; 1974.
12. Bassili, W. R.: Stewart, G. T. Epidemiological evaluation of immunisation and other factors in the control of whooping cough. Lancet 1:471–473; 1976.
13. Stewart, G. T. Vaccination against whooping-cough: efficacy versus risks. Lancet 1:234–237; 1977.
14. Curran, W. J. Drug-company liability in immunization programs. N. Engl. J. Med. 281:1057–1058; 1969.
15. Byers, R. K.; Moll, F. G. Encephalopathies following prophylactic pertussis vaccine. Pediatrics 1:437–456; 1948.
16. Toomey, J. A. Reactions to pertussis vaccine. J.A.M.A. 139:448–449; 1949.
17. Globus, J. H.; Kohn, J. L. Encephalopathy following pertussis vaccine prophylaxis. J.A.M.A. 141:507–509; 1949.
18. Doull, J. A.: Shibley, G. S.: McClelland, J. E. Active immunization against whooping cough. Interim report of the Cleveland experience. Am. J. Public Health 26: 1097–1105; 1936.
19. Kendrick, P. L.; Eldering, G.; Borowski, A. A study in active immunization against pertussis. Am. J. Hygiene 29:133–153; 1939.
20. Sako, W.; Treuting, W. L.; Witt, D. B.; Nichamin, S. J. Early immunization against pertussis with alum-precipitated vaccine. J.A.M.A. 127:379–384; 1945.
21. Sako, W.; personal communication cited by Byers, R. K.; Moll, F. G. Encephalopathies following prophylactic pertussis vaccine Pediatrics 1:437–456; 1948.
22. McComb, J. A.; Trafton, M. Z. Immune responses and reactions to diphtheria and tetanus toxoids, with pertussis vaccine, aluminum phosphate precipitated. N. Engl. J. Med. 243:442–444; 1950.
23. Watson, E. H.; Stewart, W. C. Immunization of children: a report on aluminum-phosphate adsorbed triple vaccine. U. Mich. Med. Bull. 18:311–315; 1952.
24. Anonymous. Vaccination against whooping cough. Br. Med. J. 2:454–462; 1956.
25. Volk, V. K.; Bunney, W. E. Diphtheria immunization with fluid toxoid and alum-precipitated toxoid. Am. J. Public Health 32:690–699; 1942.
26. Barrett, C. D.; Timm, E. A.; Molner, J. G.; et al. Multiple antigen for immunization against poliomyelitis, diphtheria, pertussis, and tetanus. J.A.M.A. 167:1103–1107; 1958.
27. Provenzano, R. W.; Wetterlow, L. H.; Ipsen, J. Pertussis immunization in pediatric practice and in public health. N. Engl. J. Med. 261:473–478; 1959.
28. Vaccination against whooping-cough. The Final Report to the Whooping-cough Immunization Committee of the Medical Research Council and to the medical officers of health for Battersea and Wandsworth, Bradford, Liverpool, and Newcastle. Br. Med. J. 1:994–1000; 1959.
29. Malgrem, B.; Valquist, B.; Zetterstrom,, R. Complications of immunization. Br. Med. J. 2:1800–1801; 1960.
30. Strom, J. Is universal vaccination against pertussis always justified? Br. Med. J. 2:1184–1186; 1960.
31. World Health Organization. Public Health Report No. 8:47, 1961.
32. Hellerstrom, B. O. Electroencephalographic studies in triple-immunized infants. Br. Med. J. 2:1089–1091; 1962.
33. Weihl, S.; Riley, H. D.; Lapin, J. H. Extracted pertussis antigen. Am. J. Dis. Child. 106:210–215; 1963.
34. Conner, J. S.; Speers, J. F. A comparison between undesirable reactions to extracted pertussis antigen and to whole-cell antigen in D.P.T. combinations. J. Iowa Med. Soc. 53:340–342; 1963.
35. Hall, R. D. Reactions to diphtheria-tetanus-pertussis antigen. Public Health. 79:332–339; 1965.
36. Adams, E.; Burian, V.; Kubatova, E.; et al. Reactogenicity of diphtheria, tetanus and pertussis vaccine in primary vaccination of infants and revaccination of school children. J. Hyg. Epidemiol. Microbiol. Immunol. (Prague) 11:90–103; 1967.
37. Strom, J. Further experience of reactions, especially of a cerebral nature, in conjunction with triple vaccination: a study based on vaccinations in Sweden 1959–65. Br. Med. J. 4:320–323; 1967.
38. Butler, N. R.; Voyce, M. A.; Burland, W. L.; Hilton, M. L. Advantages of aluminum hydroxide adsorbed combined diphtheria, tetanus, and pertussis vaccines for the immunization of infants. Br. Med. J. 1:663–666; 1969.
39. Cohen, H. H.; Hannik, C. A.; Nagel, J. Success and limitations of vaccination against pertussis. In Proceedings of International Conference on the Application of Vaccines against Viral, Rickettsial, and Bacterial Diseases of Man, 14–18 December, 1970. Chapter IVB. Washington, D.C. Pan American Health Organization; 1971:323–328.
40. Perkins, F. T.; Sheffield, F.; Miller, C. L.; Skegg, J. L. The comparison of toxicity of pertussis vaccines in children and mice. Symp. Series Immunobiol. Standards 13:141–149; 1970.
41. Someya, S. Studies on the prophylactic effects and untoward reactions of pertussis-diphtheria-tetanus combined vaccine. VI. Report of a field trial. Nuff. Int. Public Health 21:68–76; 1972.
42. Ehrengut, W. Über konvulsive reaktionen nach pertussis-schutzimpfung. Dtsch. Med. Wochenschr. 99:2273–2279; 1974.
43. Ehrengut, W.; personal communication to Gostling, J. V. T.; Payne, D. J. H. Whooping-cough vaccination. Lancet 2:773; 1974.

44. Miller, C. L.; Pollock, T. M.; Clewer, A. D. E. Whooping-cough vaccination: an assessment. Lancet 2:510–513; 1974.

45. Dick, G., in International symposium on combined vaccines: p 25, Basel, 1967.

46. Dick, G., in Proceedings of an international conference on the application of vaccines against viral, rickettsial and bacterial diseases in man. Washington, D.C., 1971, p. 420.

47. Dick, G. Commun. Med. 127:73; 1972.

48. Dick, G. Reactions to routine immunization in childhood. Proc. R. Soc. Med. 371–372; 1974.

49. Anonymous. Immunization against whooping cough. W.H.O. Chronicle 29:365–367; 1975.

50. Melchior, J. C. Infantile spasms and early immunization against whooping cough. Danish survey from 1970 to 1975. Arch. Dis. Child. 52:134–137; 1977.

51. Griffith, A. H. Reactions after pertussis vaccine: a manufacturer's experiences and difficulties since 1964. Br. Med. J. 1:809–815; 1978.

52. Lapin, J. H. Whooping cough. Springfield, Ill.: Charles C Thomas; 1943:155–174.

53. Sako, W. Studies on pertussis immunization. J. Pediatr. 30:29–40; 1947.

54. Dick, G. Whooping-cough vaccine. Br. Med. J. 4:161; 1975.

55. Whooping cough vaccination. Review of the evidence on whooping cough vaccination by the Joint Committee on Vaccination and Immunization, Department of Health and Social Security. London: Her Majesty's Stationery Office; 1977.

Experiences of Pertussis in the United Kingdom

C. H. Stuart-Harris

ABSTRACT

Large changes have occurred in the incidence and mortality from pertussis in the past 40 years in the United Kingdom because of changes in the birthrate and other factors such as the Second World War. Since the introduction of immunization as a matter of national policy in 1957 a decline in the annual numbers and attack rates from pertussis has occurred in all age groups including those under one year of age. In the United Kingdom, as in the United States, the protection afforded by immunizing the older children in the family to prevent infection of the unimmunized baby appears to be a major criterion of the vaccine's efficacy.

Since October 1977 an intense outbreak of pertussis, mostly among unvaccinated children under 5, has affected England and Wales. This is hardly surprising in view of a decline in the acceptance rate of pertussis vaccine since 1974 (from 70–80% to 40%). The vaccine acceptance rates and the pertussis rates are inversely correlated in several geographic areas during the present epidemic.

Much of the decline in public acceptance of vaccination in the United Kingdom is due to publicity by the Association of Parents of Vaccine-Damaged Children on possible adverse neurologic effects of pertussis vaccine. The U.K. government has agreed to the principle of compensation for damage from vaccine. The Joint Committee on Vaccination and Immunisation, concerned by the findings of retrospective inquiries, has encouraged prospective studies of adverse reactions to immunization. The Public Health Laboratory Service, through its epidemiological laboratory, has sought to monitor all such reactions since 1975. In 1976 the first comprehensive national inquiry was launched to collect data on all children under 3 years of age hospitalized with an illness covered by the label "encephalopathy," which covers infantile spasms. The study will not be complete until 1980.

The incidence of pertussis in Britain has undergone large changes in the past 40 years. Figure 1 shows that postwar epidemics from 1948 to 1954 accompanied the high birthrates of those years, but by 1956 notifications had subsided to the 1947 level (1). After the trials of pertussis vaccine carried out by the Medical Research Council's Whooping Cough Immunisation Committee (2, 3), which fully confirmed the protective effect shown in American trials of Michigan vaccine (4), immunization against pertussis with triple (DTP) vaccines was adopted as national policy in 1957. The number of notifications of pertussis fell sharply from 1958 to 1961, then more slowly with periodic rises every 3 to 4 years, and reached their lowest level in 1973. Mortality declined steadily from 1947 onwards well before general use of vaccine yet the deaths maintained a ratio of 1 per 1,000 notifications until the current outbreak, which began in 1977.

Notification rates per 1,000 children by age, case fatality rates per 1,000 notifications and deaths per million are shown in Table 1 in 4-year consolidated totals. The striking reduction in notification rates

from 1957 onward occurred in all age groups and continued until 1973. Case fatality rates also fell except in infants under 1 year of age and remained at about 5 to 9 deaths per 1,000 notifications from 1957 onwards. Most deaths of recent years have been in infants under 1 year and often less than 6 months of age when, at most, they could have had only one dose of DTP. It is in precisely such children that protection from infection in the home by immunization of older siblings was shown to be valuable in the earlier trials of vaccine. Last year the Joint Committee on Vaccination and Immunisation tried to change the start of immunization from 6 to 3 months of age so that at least some children at risk might receive three doses by the time they are 8 months old.

After 1974, following the paper by Kulenkampff et al. (5) on neurological sequelae to immunization with DTP vaccine, a group called the Association of Parents of Vaccine-Damaged Children has conducted a campaign in the United Kingdom to obtain compensation for children with alleged brain damage after vaccine. There have also been opin-
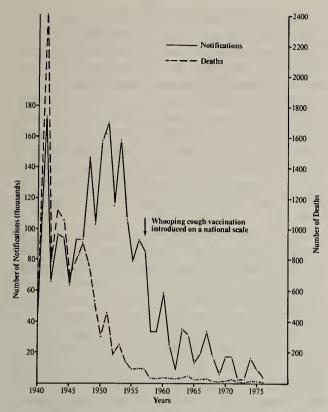
Figure 1. Whooping cough, England and Wales, 1940–1976. Reproduced with permission of Controller, Her Majesty's Stationery Office, from Report of Joint Committee on Vaccination and Immunisation (1977).

one member of Parliament, has received much publicity in the United Kingdom, with television playing a major role. Few attempts to present a balanced argument have been made by press or TV until quite recently, and the public has reacted with a sharp fall in the rate of acceptance of triple and other vaccines. By 1976 the nationwide percentage of parents accepting vaccination for their children had dropped from 70–80% to less than 40%. In different areas the acceptance rate ranges from 11% to 60%.

Whooping Cough in 1977 and 1978

A sharp rise in pertussis notifications began in November 1977 and the level has remained high ever since. Figure 2 shows quarterly notification rates in age groups for England and Wales. The rate for January to March 1978 was the highest quarterly rate since before 1960, and notifications have continued at 1,000 or more a week with only temporary decreases to 800. Seventy-five percent of the notifications have been in children under 5 years old. The older children, more of whom are fully immunized, have been relatively spared.

This dramatic reversal of the fall in incidence of pertussis suggests that vaccine had previously been exerting a true protective effect. Its partial withdrawal since 1974 has had the greatest impact on children born in that year and since. A comparison of attack rates in immunized and unimmunized children cannot be done until the epidemic ends. Noah (6) analyzed attack rates from 1972 to 1974 by comparing the proportions of fully and partly immunized children and showed the favorable

ions that pertussis vaccine should be withdrawn. It must be emphasized that no vaccine is compulsory in the United Kingdom. The only vaccine ever to have been compulsory, that against smallpox, became voluntary in 1948. The parents' campaign, supported by some doctors and at least

Table 1. Whooping Cough, England and Wales, 1944–1973

	Incidence			Case Fatality			Mortality		
	Notifications per 1000 population			Deaths per 1,000 notifications			Deaths per million population		
	Under 1 year	1–4 years	5–9 years	Under 1 year	1–4 years	5–9 years	Under 1 year	1–4 years	5–9 years
1944–45	12.1	17.7	8.4	65.7	6.99	1.05	796.3	123.6	8.8
1946–49	14.6	22.4	11.0	42.6	4.07	0.40	620.6	91.0	4.4
1950–53	19.3	28.2	15.0	15.9	1.24	0.12	307.7	35.1	1.8
1954–57	12.1	16.5	9.7	8.8	0.55	0.09	106.1	9.0	0.8
1958–61	5.0	6.1	4.4	5.3	0.46	0.07	26.5	2.8	0.3
1962–65	3.1	3.5	2.1	9.2	0.60	0.04	28.2	2.1	0.1
1966–69	2.3	3.0	1.6	8.7	0.13	—	19.8	0.4	—
1970–73	1.6	1.3	0.9	8.2	0.24	0.14	13.1	0.3	0.1

Sources—see Table 1A, populations from Registrar-General's Statistical Review of England and Wales, Part II—Tables, Population.

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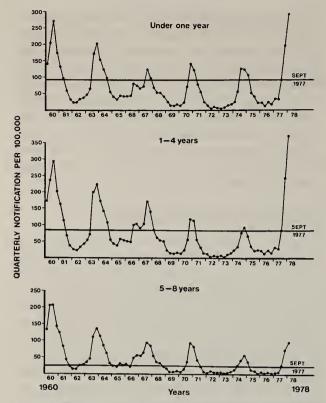
Figure 2. Pertussis quarterly notification rates, England and Wales. By courtesy of Dr. Peter Lambert, Office of Population Censuses and Surveys, London.

effect of immunization in each 6-month period. A recent study in Hertfordshire by Dr. M. Church (Table 2) shows that during 1977, children under 5 who were fully immunized had a rate of pertussis one-tenth that in unimmunized children (90% pro-tection). An analysis in general (family) practice by Jenkinson (7) showed that immunized children 1 to 5 years of age had an attack rate about one-sixth that of unprotected children (84% protection).

In Figure 3 attack rates in 98 areas of England and Wales from October 1977 to March 1978 are

compared with vaccine acceptance, expressed as the proportion of children born in 1974 who completed a full course of three injections by 1976. The attack rates in children aged 0 to 14 calculated from the mid-1976 population ranged from 67.6 to 833.3 per 100,000. Acceptance rates in the same areas as calculated for 1976 ranged from 11% to 60% of children born in 1974. A statistically significant negative correlation exists between the two rates, and there is no difference between the correlation in urban and rural areas. This strongly indicates a protective effect from pertussis vaccine during the current epidemic.

Opponents of vaccine in the United Kingdom have not been slow to suggest to the press that the current epidemic is an illusion due to diagnostic confusion with respiratory virus infection. It has been shown that there are common respiratory virus infections that may resemble whooping cough (8) and doubtless may accompany true pertussis. On the other hand, it has also been shown that *Bordetella pertussis* may be difficult to cultivate from swabs (8). Only a full serological study can adequately supplement culture methods, and is particularly fruitful in children over 1 year of age (9). Bac-teriological results in the Public Health Laboratory Service of England and Wales showed a sudden increase in isolations of *B. pertussis* that paralleled the rise in notifications in November 1977. Isola-tions and notifications have continued to move in parallel ever since. Hospital patients have yielded organisms more often than those from home. Rates of 50% have been achieved by some hospital labora-tories. The organisms have been serotypes 1.3.

Severity of Illness in the Present Outbreak

During the outbreak of 1974–1975, Miller and Fletcher (10) surveyed 8,092 notifications of per-tussis and found that 775 (10%) were hospitalized.

Table 2. Whooping Cough Notifications in Hertfordshire; Children of 5 Years and Under During 15 Months—1977 and the First Quarter of 1978

Status	Cases (No.)	Children (No.)	Whooping Cough (Rate/100,000)	Protection (%)
Fully Immunized	27 (9%)	33,753 (48%)	80.0	90%
Partially Immunized	36 (13%)	10,340 (15%)	348.2	59%
Not Immunized	225 (78%)	26,748 (37%)	841.2	
Totals	288 (100%)	70,841 (100%)	406.5	

Courtesy of Dr. M. Church, Hertfordshire Health Authority (personal communication, 1978)
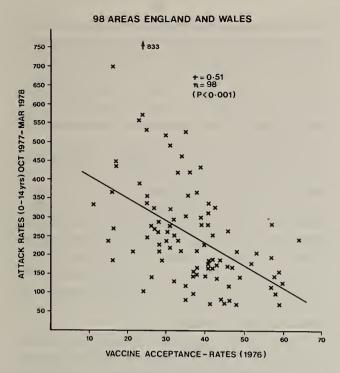
Figure 3. Courtesy of Dr. W. Dunnet, Department of Health and Social Services, London. Statistical advice from Professor J. Knowelden, Department of Community Medicine and Medical Care, University of Sheffield.

In 15 areas studied at Colindale (Pollock, personal communication, 1978) the rate of admission was 5%. In these same areas 5.9% of notified children have been hospitalized in the current epidemic.

Clinical reports from hospital cases suggest, however, that the severity has been widely variable and that the mortality so far (12 deaths) has been lower than in previous outbreaks. This can be judged by comparing the number of deaths among those under 1 year of age with the notifications per 1,000 children of like age, as an index of the case fatality rate. The total number of notifications received by the Office of Population Censuses and Surveys (Lambert, personal communication) in the first 6

months of the outbreak was 2,683 and the case fatality rate of 4.4/1,000 is only slightly lower than the rate of 5.3 in 1958–1961. Again the rate must be assessed on the whole outbreak because there is usually delay before death certification can be accurately evaluated. It seems possible that notifications are more complete in the current outbreak because of the publicity. Prophylactic studies of antibiotics have also been done.

Vaccine Risks

Surveillance of the adverse effects of all drugs and vaccines used in the United Kingdom is by the Committee on the Safety of Medicine (CSM). It receives reports from doctors recording illnesses that have occurred after drug administration or vaccination. The reports are regarded as pointers to particular hazards rather than a basis for epidemiologic inquiry. In the past 10 years, illnesses occurring after DTP vaccine have covered many clinical patterns, but interest has centered on those suggesting neurologic involvement and particularly on those with permanent residual disability. Screaming attacks, twitching, or convulsions noted by several observers in the past have been reported, though their frequency has varied greatly. In some children persistent convulsions, coma, neurological signs, and retarded development have also been found. These were the illnesses documented retrospectively in hospital cases in the United States in 1948 by Byers and Moll (11) and by Kulenkampff et al. (5) in London. Are such severe illnesses truly caused by vaccines containing pertussis bacilli or do they occur by chance in relation to the date of inoculation of vaccine? How frequently are they encountered?

The answers to these two questions are not known with certainty. It is impossible with retrospective inquiries to verify accurately either the previous state of health of small babies before inoculation or the subsequent march of events. Because of

Table 3. 1977-8 Outbreak of Whooping Cough in General Practice[a]

Age Group	<1	1–2	2–3	3–4	4–5	1–5
Year of birth	1977	1976	1975	1974	1973	1976–1973
Number at risk	87	141	138	167	140	586
Number immunized	—	43	76	91	105	315
Cases of whooping-cough (immunized in brackets)	9	24 (0)	25 (2)	42 (7)	26 (9)	117 (18)

1–5 years attack rates: Immunized 5.7%; unimmunized 36.53%
[a] Data from Jenkinson, D. Br. Med. J., 3:577–578; 1978.

this the Vaccine Complications Subcommittee of the British Joint Committee on Immunisation has initiated an important prospective study under the chairmanship of Professor Alastair Dudgeon. It is called the National Childhood Encephalopathy Study and has been directed by Professor David Miller of the Middlesex Hospital Medical School. The study is of all children under 3 years of age hospitalized with an illness defined by certain criteria as "encephalopathy." Experience shows that convulsions without sequelae, attacks of infantile spasms (West's syndrome), and convulsions of true encephalitis are far from rare. Professor Dudgeon, who is here today, may have something to say about this study, which is still in progress.

So far, the assessment of cases reported to the CSM and those from the Parents' Association shows that most of the illnesses temporally associated with DTP vaccine do not appear to be clinically different from illnesses continually occurring in children of similar age irrespective of recent inoculations. It is therefore difficult to believe they are the result of the neurotoxin that Stewart (12) claims is a component of the vaccine.

One type of illness requires mention—the convulsion. There is no doubt that convulsions sometimes occur within 24 to 48 hours of DTP vaccine injection. At that time there may be a rise in body temperature, and this is a reasonable cause for a convulsion in babies of an age when febrile fits are relatively common (13).

Hannik (14) has reported that 1 in 2,150 children inoculated with DTP vaccine in The Hague suffered convulsions, but the rate in other areas of the Netherlands was much lower. A study still in progress in one of the London Regions by Drs. T. Pollock and C. M. Miller of the Central Public Health Laboratory has now covered 154,000 inoculations of DTP in primary immunizations. Ten children have had convulsions within 1 week of inoculation, a rate of 1 in 15,000 inoculations. Two children with more serious illnesses, one with a period of staring (vacancy) and the other with infantile spasms, were encountered. Both have made a good recovery. So far, no case of permanent brain damage has been encountered in this study. The apparent rarity of such damage is also confirmed by those who have deliberately looked for and failed to find such cases (15).

Advice against using pertussis vaccine in children with certain past conditions or existing acute illnesses has been given to general practitioners and others concerned in immunization for a number of years in the United Kingdom. The vaccine's use is proscribed for any child with a personal or family history of convulsions or epilepsy, or a neonatal illness with neurological signs, or who is suffering from an acute illness. These limitations are essential in Britain at the present time when parents and practitioners alike are apprehensive over risks.

The controversy in the United Kingdom continues. Because there are still some people, doubtless well meaning, who seek to have the vaccine officially withdrawn, advocacy of the vaccine is weakened by repeated allegation of its risks. Public confidence once upset is slow to return and the nurses and doctors concerned with administering vaccines are caught by the controversy so carefully nurtured by the opponents. Agreement among us is confined to the wish for a vaccine that is completely bland, yet fully protective.

Meanwhile, the British Government has accepted the principle of compensation for disability related to the administration of vaccine and has made *ex gratia* payments to the parents of alleged vaccine-damaged children.

Summary

The experience in Britain of nearly succeeding in obtaining control of pertussis and then suffering a very large epidemic upholds the view that pertussis vaccine administered as DTP is fully protective. A partial withdrawal of pertussis vaccine has occurred as a direct consequence of a hostile public reaction engendered by allegations that the vaccine is unsafe. This has had the same effect that official withdrawal, gratuitously advised by various persons, would have. The future use of the vaccine depends on the restoration of public confidence. This may be helped by the attitude of practitioners and nurses upon whom advice on health matters ultimately depends. It seems that a need exists for health professionals to explore the best method of explaining the issues concerning immunization to the public.

REFERENCES

1. Report of Joint Committee on Vaccination and Immunisation. Whooping-cough vaccination: review of the evidence on whooping-cough vaccination. London: Her Majesty's Stationery Office; 1977.
2. Report to the Whooping-Cough Immunisation Committee of the Medical Research Council. The prevention of whooping-cough by immunisation. Br. Med. J. 1:1464–1473; 1951.

3. Report to the Whooping-Cough Immunisation Committee of the Medical Research Council. Vaccination against whooping-cough. Relation between protection in children and the results of laboratory tests. Br. Med. J. 2:454–463; 1956.

4. Kendrick, P. L.; Eldering, G. A study in active immunization against pertussis. Am. J. Hygiene sect. B. 29: 133–153; 1939.

5. Kulenkampff, M.; Schwartzman, J. S.; Wilson, J. Neurological complications of pertussis inoculation. Arch. Dis. Child. 49:46–49; 1974.

6. Noah, W. D. Attack-rates of notified whooping-cough in immunised and unimmunised children. Br. Med. J. 1:128–129; 1976.

7. Jenkinson, D. Outbreak of whooping-cough in general practice. Br. Med. J. 3:557–578; 1978.

8. Pereira, M. S.; Candeias, J. A. N. The association of viruses with clinical pertussis. J. Hygiene (Cambridge) 6:399–403; 1971.

9. Combined Scottish Study. Diagnosis of whooping-cough: Comparison of serological tests with isolation of *Bordetella pertussis*. Br. Med. J. 4:637–639; 1970.

10. Miller, C. L.; Fletcher, W. B. Severity of notified whooping-cough. Br. Med. J. 1:117–119; 1976.

11. Byers, R. K.; Moll, F. C. Encephalopathies following prophylactic pertussis vaccine. Pediatrics 1:437–457; 1948.

12. Stewart, G. T. Vaccination against whooping-cough. Lancet, 1:234–237; 1977.

13. Van den Berg, B. J.; Yerushalmy, J. Studies on convulsive disorders in young children. Pediatr. Res. 3:298–304; 1969.

14. Hannik, C. A. Surveillance of reactions to biologicals. Proc. Int. Conf. Role of the Individual and the Community in the Research, Development and Use of Vaccines. Bull. W.H.O.:55 Suppt. 133–135; 1977.

15. Griffith, A. H. Reactions after pertussis vaccine: a manufacturer's experience and difficulties since 1964. Br. Med. J. 1:809–815; 1978.

Pertussis Vaccine: The United Kingdom's Experience

G. T. Stewart

ABSTRACT

During the last 80 years or more, pertussis has declined in the United Kingdom in the rise-and-fall fashion characteristic of major childhood infections. This decline continued after a national vaccination program was started in 1957, but about 90% of the total reduction in mortality of the disease since 1920 occurred before the vaccination program began and about 85% before vaccine was available. The incidence of disease also decreased considerably and continuously during this period. Vaccines in use during the 1950s were shown then to have a significant protective effect in clinical trials, but the effect has been variable and incomplete since that time. Vaccine acceptance has fallen in recent years from over 70% to under 50% in most parts of the United Kingdom, which is now experiencing a widespread though patchy epidemic of pertussis and other respiratory infections. The partially protective effect of vaccination is again apparent, but surveillance at national and local levels indicates that 25–50% of cases have received three doses of DTP. Respiratory complications and encephalopathy appear to be extremely uncommon. The death rate is lower than in any previous epidemic and is confined mainly to risk groups in the early months of life. Reactions to pertussis vaccine are common but usually transient. Severe reactions followed by brain damage are estimated at 1:20,000–1:50,000 children vaccinated.

INTRODUCTION

Three aspects of pertussis are attracting international attention to the United Kingdom. First, there is an outbreak of pertussis, which has to be considered against the general epidemiological background of this and other infections of childhood. Second, there is widespread but diminishing use of pertussis vaccine, with possible influences on the pattern of the disease. Third, there are questions about the toxicity of the vaccine and about the relative risks of infection and vaccination. To deal with all of these questions in a short time is a tall order but fortunately one that depends less on argument than on evidence. My task here is simply to present the evidence.

In any discussion of major herd infection, we have to acknowledge as a fact of medical history that there has been a general and progressive decline in incidence and severity during the last century. This applies notably to pertussis, or whooping cough, as the syndrome of recurring spasmodic coughing with or without inspiratory whoop is often called. It applies especially when the syndrome is associated with the presence of *Bordetella pertussis*, which is sometimes but not always the main identifiable pathogen (1,2). There was a time, lasting until the 1950s in developed countries, when pertussis was still prevalent and sometimes deadly, but it is a matter of record that thereafter it began to decline, not dramatically like diphtheria but gradually like tuberculosis, measles, scarlet fever, and indeed most other childhood infections. Because of changes in the pattern and practice of notifications in different countries, the decline in incidence can sometimes be questioned, but there is no question about the decline in severity and mortality of pertussis. It is a matter not only of record but of common knowledge.

In the epidemiology of children's infections, the population at risk fluctuates sharply within short periods. The general decline of the major infectious diseases has to be viewed against the low birth rates between 1930 and 1945 and since 1965 in Europe and North America, in contrast to the high birth rates in 1918–1925 and 1945–1955. It also is a fact of medical history that malnutrition, herding, poor antenatal and obstetric care, and poor living conditions, which had persisted from the 19th century through the first half of this century, also improved more rapidly in the 1950s and that the health of children everywhere reflected this improvement. Finally, a number of medical interventions began to be made, notably with vaccination and antimicrobial drugs, which contributed for the first time in medical history an element of specific prevention and therapy of particular diseases. The question everyone at this symposium is directly or indirectly asking is whether the natural decline of

pertussis has been accelerated by the use of vaccines and, if so, whether we need to continue using them to prevent epidemics. It is usually assumed that we do.

EPIDEMIOLOGY OF PERTUSSIS IN THE UNITED KINGDOM

It is surprising in this century, when statistics, data processing, and computation generally have become exact sciences, that notification and surveillance of communicable disease remains an inexact, even primitive business. This is true in the United Kingdom as elsewhere. For lack of any other data on the national scale, we use notifications only as a guide to what is going on. In a limited way, they are valuable because they do parallel hospital admissions and isolations of *Bordetella pertussis*, though they may also reflect the rise and fall of other acute respiratory episodic illnesses, especially since the mild attacks that are now usual are difficult to differentiate from other infections (Fig. 1).

1850–1950

In the United Kingdom, notifications have declined steadily since about 1900. The pattern of decline is that of a highly communicable infection likely to affect a high proportion of susceptible individuals but conferring immunity that usually lasts for several years. Children and sometimes
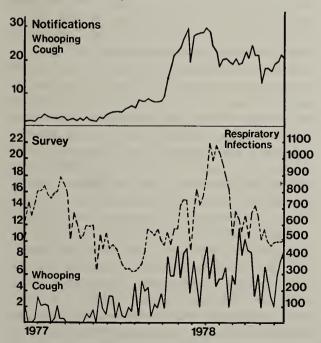
Figure 1. Notifications and surveys of respiratory infections and whooping cough in England and Wales, 1977–1978 (rates per 100,000) (Ref. 99).

adults remain infective at the end of an epidemic and pass the pathogen on to those who have escaped infection by being single children, by being absent from schools and playgroups or otherwise sheltered, or by being born after the epidemic. As a typical herd infection, pertussis spreads more widely in crowded communities like the major cities of the United Kingdom and other European countries during World War II. This may explain the striking rise that occurred during the years 1940–1945 in Europe but not in North America. After the war, the birth rate and child population rose sharply, but pertussis resumed its trend of continuous decline with a gradual widening of the interepidemic period from 2 years to its present rather curious spacing of just over 40 months between epidemic peaks (Fig. 2).

Deaths and hospital admissions fell more rapidly than notifications. The death rate fell very sharply indeed during the period of the postwar rise in notifications (Fig. 3). It is possible that antimicrobial therapy played a part in this by controlling secondary infection, but it was not the major factor. The trend of decline was already established, and the average rate over the 45 years from 1900–1945 was about 5%. In the next decade it fell about three times faster than in the preceding four decades to reach a low level of about 1 death per 1,000 cases in the mid-1950s. This level is accurately predicted by the regression line for deaths over time for the period 1930–1950 (Fig. 4), in which the constancy of decrease is highly significant. There can be no debate about the role of vaccine during these years in the United Kingdom, because except for limited trials it was simply not in use.

The United Kingdom in the hundred years after 1850, when records of morbidity and deaths became
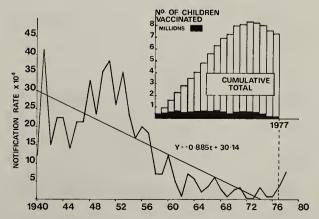
Figure 2. Notification of whooping cough in England and Wales, 1940–1978.
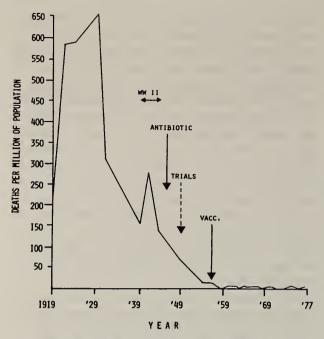
Figure 3. Deaths from whooping cough, England and Wales, 1919–1977.

available, was a scene of great demographic and social changes. The child population increased enormously, but mortality from whooping cough decreased from over 1,000 to less than 10 per million of population. The same can be said of diphtheria, measles, scarlet fever, and tuberculosis, which were equally devoid of any specific medical measures for prevention or therapy until the end of the centennial period (3). Of the total decline in recorded mortality of these diseases to date, 90% occurred before the advent of vaccination programs or antibiotics.
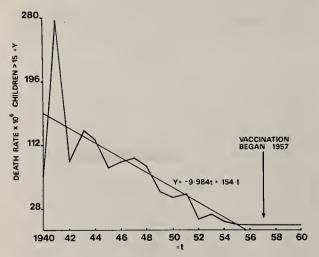
Figure 4. Deaths of children from whooping cough, England and Wales, 1940–1960.

1950–1970

Anyone who was engaged in medical practice during and immediately after World War II recalls that whooping cough was still a prevalent, highly infectious, distressing disease carrying a risk of serious complications and death in infants. The situation in the Old World (4–7) seemed to be even worse than in the United States (5) and Canada where there had been no dislocation or herding of communities through bombing and evacuation, and where a vaccine program had been adopted on a fairly wide scale by 1950 (6). Since early experiences in Britain with vaccines had been discouraging, it was natural that attention focused on the situation in America, especially on the possibility that American vaccines were more potent (4,6).

This led the Medical Research Council to plan an extensive trial of various vaccines, which was reported in 1951 (7). Unlike previous trials, this one was so carefully controlled for age, exposure, and other variables that it still ranks as one of the best. It showed, or seemed to show, that certain American vaccines were strongly protective. Unfortunately, it did not investigate the situation in the most vulnerable group, infants, nor did it show that an epidemic as opposed to a sporadic outbreak could be controlled. It was apparent too that there was an appreciable though unstated incidence of immediate reactions to the vaccine leading to withdrawals from the program. These were not investigated further, either then or subsequently (8,9). The reports of 1951 and 1956 were held to establish a few years later (8), and subsequently (6), the validity of the mouse protection test (a distinctly bizarre assay of vaccine potency), but on a once-only retrospective basis that has never been confirmed or performed prospectively in outbreaks of infection in humans.

However, on this basis, which by present standards would be unacceptable in the United Kingdom and the United States, a program of mass vaccination was started in 1957 (4) on such a scale that within 2 years about 80% of young children were vaccinated under the national supervision of the Public Health Laboratory Service (P.H.L.S.) (Fig. 2).

The results were disappointing. The P.H.L.S. reported in 1969 (1) and 1973 (9) that the vaccines used before 1968 were practically ineffective. Nevertheless, during the same period the trend of decline in notifications continued while mortality stayed at the same low level it had reached before the vaccination program began (Fig. 3). In the confused epidemiology of pertussis, this is one of the few

revealing episodes: the sting went out of the disease at a time when, whatever else was happening, the vaccines were manifestly ineffective in controlling the epidemics of 1963–1964 and 1967–1968 or the sporadic occurrence of the disease between the epidemics.

The failure of the vaccines used during the period 1957–1968 has been attributed by Preston (10, 11) to a change in serotype of *B. pertussis* from a predominance of type 1.2 in the 1950s to types 1. 2.3 and 1.3 in the 1960s. It has been confirmed that serotype 3 became more prevalent by 1968, but reference figures and denominators for preceding years are not available. Apart from this, there is considerable doubt about the role, if any, that serotype agglutinogens play in vaccine potency. In any event, one of the main manufacturers of vaccine in Britain has confirmed the presence of agglutinogen 3 in its vaccine strains since 1960 (13). Also during this period there were some changes in manufacture and standardization of vaccines (13–15), notably as the result of recognition in 1964 that all British vaccines used previously were below international standards of potency. So there are some serious inconsistencies: vaccines said to be clinically efficacious in 1957 were below American and international standards of potency, while later vaccines of higher potency were ineffective clinically and epidemiologically.

1970–1976

By 1970, all vaccines used in the United Kingdom had been altered to include serotype 3 and to raise the potency (4,9). Despite the adverse reports of the P.H.L.S. (1,9) there had been no substantial fall in vaccine acceptance (4,13,16) for the child population (Fig. 2). Even so, the outbreak of 1970–1971 was not prevented. And it showed, for the first time, a relatively higher incidence in infants and toddlers, who for the first time in 20 years were a much smaller part of the child population because of the continuing fall in the birth rate. The number of cases in the United Kingdom in the 1970–1971 outbreak probably exceeded by far the notified total of 38,178. There were 41 deaths, mainly in the 0–2 year age group, of whom about 1.25 million (about 80%) had been fully vaccinated during the preceding 2 years. The annual report of the Department of Health and Social Security for 1970 makes the point that the "improvement" of the vaccines in routine use was obviously insufficient to prevent the epidemic, which continued through 1971 on the

same scale as in 1967–1968 before the "improvement" had been made (16).

In 1974–1975, there was another outbreak of pertussis in most of the urban areas of the United Kingdom amounting to 27,647 notified cases with 27 infant deaths. By this time vaccination acceptance had fallen steadily from 713,440 in 1971 to 300,368 (4) in 1975. The decline of acceptance has continued since that time (Fig. 2).

1977–1978

Since 1960, the interval between epidemic peaks in the United Kingdom has been just over 40 months. A new epidemic was therefore expected and actually began on schedule in the fall of 1977, reaching what we hope has been its peak in mid-1978, though the fall-away in notification since then is slower than usual (Fig. 5), having risen in September at the end of the school holidays.

The epidemic, insofar as one can assess it by notifications (Table 1), is more extensive than those of 1970–1971 and 1974–1975 but appears to be much less severe in that the number of deaths is the lowest ever recorded in the United Kingdom (17) (Table 2). The great majority (83%) of deaths since 1965 have been in social classes IV and V. In the recent epidemics this unfortunate differentiation is more marked than ever. Several of the children who died had severe congenital lesions. To date, 10 deaths have been reported in England in 1978, though only 3 have been confirmed bacteriologically. All of these deaths were in infants, mostly below 6 months of age and therefore much too young to have completed vaccination. There have been no deaths in Scotland and hospital admissions so far have not disclosed any excess of severe cases,
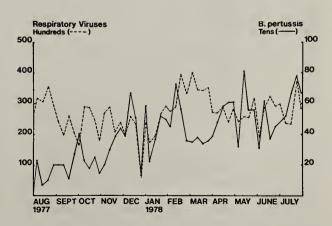
Figure 5. Isolates of *B. pertussis* and respiratory viruses, England and Wales, 1977–1978 (Ref. 27).

Table 1. Whooping Cough: England and Wales, 1970–1978[a]

	Age Groups (years)	Average 1970–73	Epidemic 1974–75	Epidemic 1977–78
Incidence	Below 1	1.6	4.5	8.5
(Notifications per	1–4	1.3	2.9	11.2
1,000 children)	5–14	0.9	0.7	2.2
Deaths per 1,000	Below 1	8.2	8.2	3.0
notified cases	1–4	0.24	0.13	0.1
	5–14	0.14	0	0
Deaths per million of	Below 1	13.1	39.6	25.6
child population	1–4	0.3	0.8	1.1
	5–14	0.1	0	0

[a] References 17 and 99

though there is as always an excess of cases from socially deprived areas.

The overall distribution is patchy, geographically and temporally. There are pockets of high incidence in the London area and parts of the Midlands and north, but some extensive and populous areas have reported few or no cases. In the larger cities, cases seem to be present in some localities and absent in adjacent ones. This is why routine returns and overall statistics are incomplete and misleading unless supplemented by shoe-leather activity in the form of visits to homes and schools to see cases, interview teachers, parents and doctors, and see what is happening. This is what I have been doing in Glasgow and elsewhere.

Table 2. Deaths with Whooping Cough as Main or Underlying Cause, England and Wales 1965–1978[a]

Year	Age (years)				Total
	<1	1–2	2–4	>4	
1965	15	5	1	—	21
1966	22	1	—	—	23
1967	24	3	—	—	27
1968	15	—	—	—	15
1969	4	1	—	1	6
1970	13	—	1	1	15
1971	22	3	—	1	26
1972	2	—	—	—	2
1973	2	—	—	—	2
1974	12	1	—	—	13
1975	11	1	—	—	12
1976	1	1	1	—	3
1977	6	2	—	—	8
(1978)[b]	9	—	1	—	10
Totals	158 (86%)	18	4	3	183
Social class IV–V	132	14	4	1	151 (83%)

[a] Reference 99
[b] January 1–September 8, 1978

The apparent extent of this epidemic, as judged by national notifications, together with the undoubted fall in vaccine acceptance suggest that lack of vaccine has made the child population more susceptible to whooping cough. If this were so, attack rates of whooping cough should be highest in areas where vaccine acceptance is low. This was not so in 1974–1975 when these two variables were not significantly correlated either in our detailed district studies in Glasgow or in the figures for the United Kingdom as a whole (18). We found then and are finding again now that a substantial proportion of notified cases are occurring in children who have been fully vaccinated (Table 3), not only in older school children whose immunity might be said to have declined but also in some of the younger preschool children.

It has to be remembered that under natural conditions immunity to this and other infections results from previous exposure or previous attack, making the cohorts of children born in or around epidemic years in areas with high attack rates more resistant to whooping cough in subsequent outbreaks (18). Routine returns like notifications are not always reliable for this kind of assessment, nor are the recollections of parents, doctors, and clinic staff. It is essential to check vaccination status from original records, if available, and to establish working record linkages. There is no national system in the United Kingdom to provide such linkage officially, so it has to be done unofficially. In Glasgow, official returns show that information about vaccination is lacking in almost half of all notifications. In the other half, cases are divided almost equally between the fully vaccinated and unvaccinated cohorts (Table 3) in hospital as well as in nonhospital cases (Table 4), as in previous epidemics. This is true of other parts of Scotland

Table 3. Whooping Cough in Glasgow 1977–1978. Population: 1,105,645 (0–15 years: 277,300)

Year	Notifications		Vaccination Status			
	Total	Corrected	Complete	Incomplete	None	Unknown
1977	321	281	77	18	108	78
1978[a]	475	458[b]	128	26	183	121
Total	796	739[b]	205 (28%)	44	291 (39%)	199
Subtotals		609[c]	205 (34%)	44 (7%)	161[c] (26.4%)	199 (32.6%)

[a] Until September 29, 1978.
[b] Incidence = 200/100,000 children at risk
[c] Total adjusted to 609 to allow for 130 below age for completion of vaccination

besides Glasgow. Indeed in surveys in the United Kingdom and elsewhere it has been found repeatedly that 40% or more of cases of whooping cough occur in fully vaccinated children (4,9,19–23). Bennett (24) reported a similar finding in Australia in 1974, acknowledging, as I do, that adequate records of what happens in the community at large are lacking. As far as I can see, the same criticism can be applied in most countries, even in the United States (Table 5), where some outbreaks have been well investigated in recent years because they have been institutional.

It might well be that one of the important messages to be transmitted from our transactions here is that in the entire field of communicable diseases, assessment and control are gravely impeded by lack of denominators and by incomplete assessment of both the independent and dependent variables. In other words, much of the precision which is expected of epidemiology is sadly lacking. To some extent, one can overcome this difficulty in one's own locality by visits to homes and schools and to as many notified cases as possible. By doing this, I have been able to confirm what I said before (23) of the 1974–1975 outbreak—that whooping cough tends to be more prevalent and more severe in deprived areas of the city from which hospital admissions are almost exclusively derived. In these areas, cases of whooping cough, especially in the 1–4 age group, are mainly among the unvaccinated. They are also often neglected in other ways and in infancy account for 80% of deaths and hospital admissions (Tables 2 and 3).

But there is more to be said than that. If we examine the distribution of whooping cough in 24% of the population of the United Kingdom in urban cluster areas matched within groups but contrasting between groups for demographic variables (Table 6), we find that there are areas with unfavorable characteristics where prevalence is relatively low and other areas with more favorable living conditions, such as cluster 1, where prevalence is high—not so high as in the major industrial cities of England (cluster 19) but much higher than in Glasgow (cluster 27), where living conditions are worse. This difference cannot be attributed to the fall-away in vaccination, because the excess cases in cluster 19 are in children aged 5–14, the age group 0–4 being below the national mean for 30 clusters. In the Shetland Islands (population 21,000) there was an outbreak of 64 cases (Table 7) beginning and spreading first among schoolgirls in a remote part of these lonely islands where living conditions

Table 4. Whooping Cough in Glasgow 1977–1978.[a] Population: 1,105,645 (0–15 years: 277,300)

Treated at	Vaccination				
	Complete	Incomplete	None	Not Known	Totals
Home	191 (158)	32 (34)	201 (225)	144 (151)	568
Hospital	14 (47)[b]	12 (10)	90 (66)	52 (45)	168
Totals	205 (28%)	44	291	196	736

[a] Until September 29, 1978
[b] (Expected no.) p < 0.01

Table 5. Whooping Cough United States, 1977–1978[a]

April 1977: Bridgton, Massachusetts
    4 children, all vaccinated plus 2 parents
May–June 1977: Decatur, Georgia
    30/94 grade 3 children and contacts
    18/75 DTP × 3 + booster (60%)
    12/19 DTP × 3
April–August 1977: Atlanta, Georgia
    75 confirmed "cases" in 56 households
    12 asymptomatic
    22 below 5 months
    17 DTP × 3 + booster(s) (23%)
        Total 109: 39 fully vaccinated for age = 36%

[a] Reference 25

Table 6. Cluster Analysis of Prevalence of Whooping Cough in Urban Areas of the United Kingdom (1977–1978)

Demographic Variables [a]	Urban Cluster Areas [b]					
	1	15	19	20	27	30
% U.K. population	4	2	8	2	2	3
0–4 age group	0.293	0.066	−.016	−.364	0.272	−.608
5–14 age group	0.386	−.079	0.496	−.377	1.064	−1.187
Large families	−.195	0.652	1.264	0.407	2.755	−.014
Overcrowding	−.506	0.230	0.165	0.962	5.215	1.285
Unskilled labor	−.878	0.428	1.047	2.596	1.840	0.315
Unemployment	−.853	−.294	1.187	0.503	3.166	0.101
Whooping cough prevalence [c]	7	5	9	3	4	6

[a] Reduced to a common scale with national mean adjusted to 0 and mean deviation ± 1

[b] Ref. 98

[c] No. of 2-week periods with notifications above national mean/percentage of U.K. population

and health indexes are generally good. The first case was a schoolgirl of 15, fully vaccinated, who had returned from a visit to Central Scotland, where whooping cough was prevalent. There has been no outbreak in the neighboring islands of Orkney, nor in adjacent parts of the mainland.

With respect to notifications, I find that there are major variations in habit and practice. Some doctors notify all suspected cases, others notify very few. There is a measurable tendency to notify unvaccinated cases more often than vaccinated ones (Table 8). In general practice bacteriological confirmation is rare. In some practices whooping cough is never notified in an unvaccinated child.

These findings exemplify the chance elements arising from speculative behavioral, geographic, and seasonal variables that we cannot predict or compute. It was obvious in Glasgow during some periods of good weather that healthy children playing outdoors escaped disease even when there were bacteriologically proven cases in the same overcrowded tenement dwellings. In bad weather, especially in schools and playgroups, the secondary attack rate in this epidemic, as in the last one (23), rises quickly to 50% or higher in vaccinated and unvaccinated contacts. The only children to escape

whooping cough under these conditions are those who have already had it. If there is a young baby in the house, the disease is sometimes alarming, but in older children complications are rare and have been so for many years (26).

In focusing our attention on the whooping cough syndrome we should not overlook the possible contribution of the croupy respiratory viruses (1,2), especially adenoviruses, parainfluenza, and respiratory syncytial virus, and the high incidence in the United Kingdom during the past year of several forms of acute respiratory disease associated with them (27). There is undoubtedly overlap in diagnosis in each direction, but the incidence of these other infections collectively and in some areas individually is much higher than that of pertussis (Fig. 5). So is the incidence in 1978 of measles and rubella. Also, the persistence of wintry weather through the summer has made this a bad year for outdoor living, and the summer seasonal decline in respiratory infection has been much less marked than usual.

Respiratory infections of this nature, transmissible directly from person to person, are always influenced by crowding in homes, nurseries, playgroups, schools, and other places. If persons remain infective during interepidemic periods, as in per-

Table 7. Whooping Cough in Walls[a] (Shetland)

	No. of Children at Risk		
	With Whooping Cough	Without Whooping Cough	Total
Vaccinated	46(47)	39	85
Not vaccinated	18	14	32
	64	53	117

[a] By courtesy of Dr. R. K. Ditchburn

Table 8. Notifications of Primary School Children with Whooping Cough According to Vaccination Status (School and Household Survey in Glasgow)

	Notified	Not Notified	Total
Vaccinated (DTP)	48	65	113(64%)
Not vaccinated	51	13	64
Total	99(56%)	78	177

$$X_y^2 = 21p \ll 0.01$$
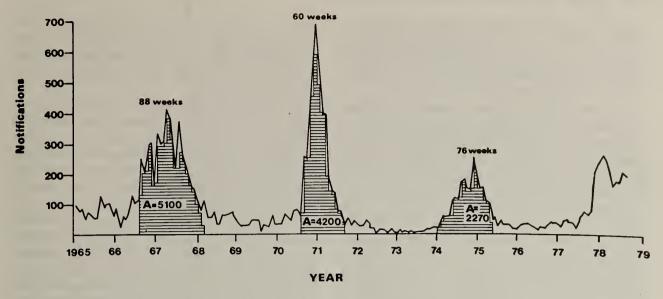
Figure 6. Notifications of whooping cough in Scotland in 4-week periods.

tussis (Fig. 6), small outbreaks can occur at any time and erupt into epidemics when the buildup of infectives coincides with the introduction of new susceptibles into the community. The demographic distribution of this type of disease in the United Kingdom during the interepidemic periods is mainly in the crowded slums, new and old, of industrial cities. As infectives in these slums build up through the epidemic threshold, infection diffuses out into the rest of the community through play-groups (28), hospitals (29), and schools, which are now comprehensively populated as a result of legis-lation since 1974.

From limited incidents and samples (19), it seems likely that older children and adults can act, symp-tomatically or otherwise, as carriers in this process of diffusion. This is perhaps the main mechanism nowadays in maintaining pertussis and other infec-tions (18,19,20,23). It is reasonable to suppose that with a lower birth rate, smaller families, and im-provements in living conditions and in the general health of children, whooping cough like other major diseases will continue to decline. However, pertussis as a relatively mild infection will probably persist, because there is now a greater admixture of children from all social classes in comprehensive schools and playgroups.

TOXICITY OF PERTUSSIS VACCINES

Pertussis vaccine is not accidentally or occa-sionally toxic. It is intrinsically toxic. It has adju-

vant properties and contains several toxic sub-stances, some of which may be identical or closely related to the toxins released by *Bordetella per-tussis* in live infections and responsible for symp-toms of whooping cough. These toxins include some substances that are pharmacologically very active. In experimental animals they cause marked alterations in heart rate, rise in blood insulin, in-creased sensitivity to histamine, and a blockage of response to epinephrine (30–35).

Except for the rise in blood insulin and fall in blood sugar (36) these effects have not been con-firmed in man, but it would be exceptional pharma-cologically if these toxins did not evoke reactions similar to those in animals. Some of the effects in animals accord well with the symptom patterns re-ported consistently in infants and children ever since pertussis vaccines first began to be used in 1930: shock, pallor, collapse, tachypnea, apnea, tachycardia, spasms, convulsions, coma.

Neurotoxic reactions, first described in 1933 by Madsen (37), who was an enthusiastic user of the vaccine, conform to a pattern which, in a review of 10 year experience of the vaccine, Byers and Moll (38) attributed in 1948 to encephalopathy with dila-tion of the cerebral ventricles. This view was sup-ported at that time by Globus and Kohn (39) and others, but Miller and Stanton (40) in a later review (1954) of cases seen by them and reported up to that time, considered that anaphylaxis was the com-mon factor. To anyone who administered pertussis

vaccine at that time, this was a likely explanation (41,42), as sudden or delayed shock with extreme "marble" pallor, stillness, apnea and unconsciousness—the so-called white syncope or white attacks —were not uncommon (43).

The best records of these attacks are personal communications and reports (44) from doctors describing such incidents following injections of DTP, which on some occasions they had given to their own children. Fortunately, these reactions are transient and may leave no aftereffects, but usually they were frightening enough to discourage a subsequent injection which, as we now know, may be followed by more severe reactions and very occasionally by sudden death (37–44), as in the anaphylactic shock reported by Werne and Garrow in identical twins (41). Nowadays, white syncope is uncommon but still occurs and is recognized by most parents if not by all doctors as an absolute contraindication to further injections of pertussis vaccine. This precaution was emphasized strongly by the Section of Pediatrics of the American Medical Association at a meeting at Western Reserve University in 1948, following a report by Toomey (45) about 38 "well authenticated" reactions to vaccines made by seven commercial laboratories.

Until 1950, reports on toxicity emanated mainly from North America, where standardized batches of pertussis vaccine began to be used fairly widely (38–42,45,46). It was not used much in other countries until 1950 or later. The first major adverse reaction reported in the United Kingdom (47) was the death in 1949 of a male twin after an 8-hour screaming fit. Increasing use after 1950 was followed by reports from different countries on neurotoxicity (48–52). The most comprehensive was that of Berg (53), who was the first to present a series in which mental defect was associated with prior reactions to pertussis vaccine.

Meanwhile, an impression of safety was conveyed by the trials of pertussis vaccine reported by the Medical Research Council of the United Kingdom in 1951, 1956, and 1959 (7,8). All the reactions mentioned above (i.e., screaming fits, pallor, and convulsions) were noted, and were described in the 1956 report as severe in one infant in every hundred who were vaccinated. In the followup report of 1958 (8), the incidence of convulsions within 72 hours of injection in 16,000 infants who each received three injections was 1:2,666 but it was stated that in each series there was no evidence of permanent brain damage or other sequelae. In retro-

spect, it seems unlikely that the followup was thorough enough to warrant this assertion, especially since it did not apply to infants who were withdrawn from the trial for unstated reasons after one or two injections.

In 1960, Strom (55) estimated the incidence of neurotoxic reactions in 215,000 children vaccinated in Sweden as 1:6,000; convulsions were 1:9,000, encephalopathy 1:20,000 and deaths 1:54,000. His findings, were challenged by Malmgren et al. (56), who claimed that the experience of physicians in Sweden was a rate of neurotoxic reactions as low as 1:50,000 but conceded that further vaccine should be withheld if there was even a transient reaction. Strom responded by stating that physicians generally were unaware of, or did not often report, adverse reactions to vaccination. In subsequent publications (57) he maintained that the incidence of neurotoxic reactions in Sweden from 1958–1965 was 1:6,000–1:3,500, though he did not state the frequency of predisposing factors or alternative diagnoses in the cases of his series. Also from Sweden, Gainstorp (58) suggested that "in rare cases" triple vaccine gave rise to infantile spasms, a view that had been expressed already in the United Kingdom by Jeavons and Bower (59), who were impressed at an early stage in their investigation by the "frequency of a history of immunization immediately prior to the first spasm." I have reason to believe (60) that both authors still hold this view after 14 years of further experience in their separate neurological assessment centers.

One of the difficulties in assessing reports during this period is that most initial reactions occur at home and are not seen by doctors until some time after the event and are rarely reported as such. Dick and his colleagues (61) observed 24 major reactions, all but one occurring in infants below 6 months of age. There was a general impression that neurotoxicity was more likely to occur in children with preexisting neurological lesions or with a family history of epilepsy. Lennox, an expert on epilepsy, wrote (62) that "efforts to prevent pertussis may precipitate brain damage and epilepsy." But there was no solid evidence until Livingston (63) observed 10 convulsions immediately after giving vaccine to 96 children with a history of febrile convulsions. There were no immediate convulsions in 298 children with idiopathic epilepsy but a temporary increase in fits in 284 with epilepsy secondary to other disorders.

In contrast to these reports by individual ob-

servers, the health authorities in the United Kingdom (4,54,65), United States (66,67) and internationally (68–70) seemed convinced that adverse reactions were transient and that the danger of brain damage was remote. Nevertheless, it is on record that strenuous attempts were still being made in the 1960s to lessen the toxicity and standardize the usage of the vaccine (6,31,71), though it is apparent also in some of these records (72) that this was no easy task. Under international agreement (69), vaccines had to be sufficiently potent to protect mice against intracerebral challenge by mouse-virulent strains of *B. pertussis*. What relationship, if any, this unnatural experimental model has to human whooping cough is doubtful. Since the toxins cannot be separated from the immunogenic antigens of *B. pertussis*, the standardization procedure imposes extraordinary constraints upon research. By definition a detoxified product is likely to lose mouse-virulence and disqualify itself for further testing. The predictable result is that pertussis vaccines are undoubtedly effective in protecting mice from the specific encephalomyelitis produced by intracranial *B. pertussis* injection, but are not necessarily on that account either protective or nontoxic in children.

In any event, after a short lull a series of reports about neurotoxicity began to be published in the 1970s (73–80), leading in 1976 and subsequently to a more controversial debate, especially in the United Kingdom, about the relative risks of vaccination and whooping cough itself (22,23,81–83). It is relevant to recall the work of Ehrengut (74), whose studies on neurotoxicity in Hamburg, combined with general experience in the area, led that city to remove pertussis from its routine vaccination schedule. This decision in Hamburg was followed by a similar decision in other provinces of West Germany in 1970. The action did not halt the general decline in the incidence or severity of whooping cough in that country (80). Ehrengut had traced the course and pattern of reactions in some detail, but the nature of the lesions associated in recent years with vaccine encephalopathy did not receive detailed attention from pediatric neurologists until 1974, when Wilson (75) and his colleagues reported a study of 36 cases referred to them for assessment and treatment. That same year an epidemic occurred in the United Kindom under conditions that prompted several investigators, including me, to reexamine the disease and the vaccine (4,18,22,23,78,82,83).

Current Studies of Toxicity of Pertussis Vaccines in the United Kingdom

As far as I know, three major studies are under way in the United Kingdom on the toxicity of pertussis vaccines.

1. The National Childhood Encephalopathy Study (N.C.E.S.) started on July 1, 1976. This is a 2-year prospective study of children hospitalized with various neurological conditions. It involves reporting of cases, followed by direct inquiry to all concerned about antecedent events including any vaccinations. Included in the survey are all cases of encephalopathy, unexplained loss of consciousness, prolonged or complicated fits, and spasms. Excluded are all cases of short or uncomplicated fits and neurological conditions where alternative diagnoses have been confirmed and there is no mention in the protocol of screaming fits, apneic attacks, or shock (81).

2. A prospective study is being made by the Central Public Health Laboratory (C.P.H.L.) at Colindale of 80,000 children who have received triple vaccine during recent years. This study deals essentially with reports of adverse reactions after each of the three injections given between 3 and 14 months of age, which is the immunization procedure in Britain.

3. A Combined Study by the Committee on the Safety of Medicines (C.S.M.) on reactions reported in the past, on brain-damaged children, and on reactions reported currently, is a consolidation since 1977 of three preceding independent studies, including my own. Essentially it consists of detailed examination of cases and of relevant control and background data by a panel of epidemiologists, pediatricians, neurologists, and others assisted by the medical secretariat of the C.S.M. The main data file is now in the large computer at the University of Glasgow, but all case records are sent to the C.S.M. for completion and assessment.

The N.C.E.S. has not yet reported. The C.P.H.L. study has been mentioned already by Stuart-Harris (84) showing, I imagine, a very fair estimate of the incidence of adverse reactions occurring immediately after administration of triple vaccine under well-controlled conditions, i.e., with all contraindications and exclusions meticulously observed.

The C.S.M. study is also incomplete and still unreported, but with their agreement I am able to quote findings on the cases I reported to them and also outline data about cases reported to them

under their yellow card system by other doctors between 1964 and 1976. Under this system, doctors are requested to notify any unexpected or severe adverse reaction to a drug or vaccine confidentially to the Committee. Further investigation of the occurrence, if appropriate, is instituted by the Committee's Secretariat and medical advisers. In addition to the routine yellow card data, my own cases are coded under 110 variables, each with up to 9 subvariables. These are entered into the computer, retrieved as required for updating, and printed out as cross-tabulations (Tables 9–16).

General Incidence of Reactions in the United Kingdom

We are not concerned here with minor and transient reactions, which have always been very common (9,43,45,52,57,76,85) after injections of triple or pertussis vaccine in adults (29) and children. Restricting our attention to reactions that are conspicuous enough to be reported to the C.S.M. on a yellow card or to be followed by medically confirmed permanent brain damage, we find (Table 9) that neurologically and otherwise the symptoms and sequence of events conform to the general pattern reported in individual cases and in smaller series between 1933 and 1974.

A minimum estimate of life-threatening reactions, made from doctors' reports to the C.S.M., would be at about 300 of 8 million children vaccinated with DTP. There were 15 deaths (1:513,829) and at least 300 neurotoxic or other reactions associated occasionally with fatality, i.e., approximately 1:26,000 in the period 1966–1978.

But notifications to the C.S.M. are notoriously incomplete. Their own estimate, based on drug monitoring, is that only about 10% of reactions are reported on yellow cards. There is no indication yet from the C.S.M. of the number of reactions followed by brain damage, though that aspect is under close investigation by an expert committee whose report, along with the National Childhood Encephalopathy Study, should in due course give useful information.

If all the cases reported to me, and through me to the C.S.M., are genuine, the incidence of brain damage could be much higher than official estimates: say about 1:50,000 children vaccinated on the basis of 250 cases so far investigated by me. With about 1,000 cases awaiting investigation, many of which are symptomatically similar, the real incidence is probably higher (Table 10), somewhere between 1:10,000 and 1:50,000. This may be as near as we can go in our estimates, for there is no sharp endpoint in brain damage. Minor degrees are common, far too common and far too diverse etiologically to be considered here (7,8,13,23,29,32–36,43, 55,76,85). So far, we have not been able to associate reactions with any particular batches or manufacture of vaccine, but we can say that no vaccine manufactured in Britain is free from toxicity.

Convulsions

The most striking reaction is the convulsion, which is most frequently reported in brain damaged children (Table 11). The main end result of brain damage is mental defect or handicap, but this is not significantly associated with frequency or severity of convulsions (Table 12). In some infants,

Table 9. Reports of Reactions to Pertussis Vaccines[a]

	No. of Reports		Total	Deaths
	1966–74	1975–78		
No. of children				
vaccinated	6,347,445	1,360,000	7,707,445	
Neurotoxic				
reactions [b]	132	167	299	10
Cot deaths	2	0	2	2
Anaphylaxis,				
hyperpyrexia	20	0	20	2
Other reactions	423	195	618	1
Total	577	362	939	15
Incidence,				
estimated	1:11,000	1:3,750	1:8,200	1:513,829
Neurotoxic reactions,				
estimated	1:48,000	1:8,143	1:25,777	1:770,746

[a] As reported by doctors to Committee on the Safety of Medicine (yellow card reports)
[b] Convulsions, 148; encephalopathy, 35; screaming, 44; coma, unconsciousness, 5; paralysis, 6

Table 10. Estimated Incidence of Brain Damage Following Neurotoxic Reactions to DTP Given to 13 Million Children, United Kingdom, 1957–1976

Cases Reported (No.)		Corresponding incidence
250	Confirmed	1:52,000 (19 per million children)
350		1:37,142 (26 per million children)
500	Under investigation	1:26,000 (38 per million children)
750		1:17,333 (57 per million children)
Total reported (all sources): 2000		1:6,500 (154 per million children)
Total reported to author: 1300		1:10,000 (100 per million children)

Table 11. Time of Reaction to DTP by Type of Reaction (271 Reactions in 197 Children with Permanent Brain Damage)

Time	Type of Reaction				
	Screaming	Convulsions	Collapse	Other Signs [a]	Total
0–4 hours	19	18	3	29	69
5–25 hours	25	35	5	39	104
1–3 days	4	13	1	7	25
≥4 days	8	17	3	6	34
Uncertain	12	4	5	18	39
Total	68	87	17	99[a]	271

[a] including one or more of named reactions
These children are all brain damaged. The reactions enumerated are those reported after their first or subsequent injections of vaccine.

convulsions followed the pattern of West's syndrome (infantile spasms) (59,75), for which no specific cause is known.

Convulsions can, of course, occur for many reasons and in many conditions (62,63,77,88,90). Those who believe that pertussis vaccines are not neurotoxic and those who are rightly critical of retrospective epidemiology would say that an infant who has a convulsion after vaccination might by chance be due for one on that day for other reasons (4). This argument is entirely reasonable and calls for a critical examination of that contingency.

Probability of Convulsions in Infancy

There are wide variations in estimate of the incidence of convulsions in infancy by different observers during the past 30 years. Between 1942 and 1954, convulsions were commonplace and about 20% were lethal. Since then, the incidence has dropped sharply (86). A survey in general practice (87) in the United Kingdom reported a cumulative

Table 12. Present State of 197 Children with Brain Damage Reported After Vaccination

Convulsions	Mental Handicap		
	Yes	No	Total
Yes	88(85) [a]	14	102[b]
No	77	18	95
Total	165	32	197[c]

[a] Expected figure in parentheses, null hypothesis
[b] Uncontrollable by drugs in 49
[c] Helpless or unmanageable, 41
This table says that, of the 197 children, 165 have mental handicap and that 88 are having convulsions. The 32 who are not mentally handicapped are handicapped in other ways, usually physically.

incidence of 3.5/1000 children per year aged 0–4 years. Van den Bergh and Yerushalmy (88) gave a higher figure (6.9/1000) for the first year of life in California, but Kurland (89) in New York gave a lower figure (2/1000 per year) during the 0–4 year age span. The Collaborative Perinatal Project (90) organized by the National Institutes of Health reported a frequency of 2 febrile convulsions (F.C.) per 1,000 infants per year in the first 6 months of life and about half that frequency of nonfebrile convulsions (N.F.C.). Griffith (91) suggested a frequency of 3.7–11.0 first convulsions during the period 6–18 months of age. Harker (92) reported a cumulative incidence of 15/1000 in the first 16 months of life. Including Harker's high figures, we might say that the consensus frequency during the period of vaccination in the United Kingdom (2–14 months) ranges from 2/1000 to 15/1000. It is interesting that in two of the American studies (88,90) the relative frequency of convulsions in the first 6 months of life seems to be higher than in Britain.

If we assume that none of the convulsions in this range is due to vaccination, the problem is (a) to compute the probability that a convulsion occurring after any one of three or four injections of triple vaccine is a coincidence; and (b) given this coincidence, to determine the probability of a second, third, or fourth coincidence. This can be calculated by letting x = the number of convulsions occurring between 2 and 14 months of age in any child. If convulsions occur independently of each other, then x can be assumed to follow a Poisson distribution with mean θ. From the frequencies quoted above, the extremes of the range of x would be 0.002–0.015. It is convenient and realistic to regard as a coincidence the occurrence of a convulsion within 3 days of vaccination and not caused by the vaccine. From the model, the probability of one coincidence, two coincidences, and so forth can be calculated as follows:

$$
\begin{array}{cccc}
 & 0 & 1 & \geq 3 \\
P(x) = & 0.997 & 0.002991 & 0.000001
\end{array}
$$

if $\theta = 0.003$

Probability (one coincidence) =
$$7.39 \times 10^{-5} = 1{:}13{,}500$$

Probability (two coincidences) =
$$7.32 \times 10^{-9} = 1{:}136{,}000{,}000$$

if $\theta = 0.01$

Probability (one coincidence) =
$$2.47 \times 10^{-4} = 1{:}4{,}000$$

Probability (two coincidences) =
$$1.96 \times 10^{-7} = 1{:}5{,}000{,}000$$

This means that if spontaneous convulsions occur at the lower end of the observed range, the chance of concurrence within 3 days of vaccination (1:13,500) is about half of the rate observed by Ström (1:6500) (55) and in more recent studies (93,94). At a higher frequency ($\theta = 0.01$), the probability of a spontaneous occurrence 1:4000) is well within the observed range. Convulsions occurring again, but only after a second vaccination, are highly unlikely to be coincidence because, regardless of the frequency of spontaneous convulsions, the chance of a coincidence within 3 days is one in several million.

It seems possible that children having spontaneous convulsions at the higher frequency may be those who, because of a low convulsive threshold or for other reasons, are likely to have febrile convulsions (86,95–97). Those at the lower end, presumably with a normal convulsive threshold, are only half as likely to have a spontaneous convulsion within 3 days, but the possibility cannot be disregarded as in the case of a convulsion in the same child after a second vaccination. With third and subsequent injections, the possibility of coincidence is extremely remote.

Convulsions are not always noticed in infants between 2 and 6 months of age, raising the possibility that in the absence of contraindications (Table 13) convulsions observed only after second or subsequent vaccinations are provoked by the vaccine. Other possibilities also have to be considered, for instance that the occurrence of a first convulsion makes the child more likely to have a second. This is covered by the low probabilities of a second convulsion at either frequency, which virtually exclude coincidence even if the conditional probability is tenfold. Convulsions occurring in less than 3 days are proportionately less likely to be coincidences.

Interpretation of Adverse Reactions

Of 197 cases of brain damage investigated in detail, convulsions account for nearly one-third of

Table 13. Contraindications to Vaccination in 197 Children Receiving DTP or Pertussis Vaccine

Reacting to	Family or personal history		Total
	Any contraindication	None	
1st injection only	43	18	61
One injection only, excluding first	35	25	60
2 or more injections	51	25[a]	76
Total	129	68	197

[a] Vaccination contraindicated in these children also by occurrence of reaction

A family or personal history of convulsions, epilepsy, or neurological abnormality is usually regarded as a contraindication. Despite the presence of scheduled contraindications, 129/197 children received vaccine, 86 on more than one occasion. In 68, there were no contraindications originally, but 25 reacted adversely to vaccine.

the observed reactions to the vaccine (Table 11). Most of these were within 5–24 hours, which means, on the Poisson model, that even a first convulsion within this period is unlikely to be a coincidence unless the child was already predisposed. Screaming fits were almost as frequent. Like the other signs (arrest of development, collapse, irritability, vomiting, paralysis), these also were usually observed within 24 hours. In most cases contraindications were present (Table 14). In 76 cases, second injections were given despite clear contraindications, which in 25 cases included adverse reactions to previous injections of vaccine (Table 13).

In 103 of 197 cases (Table 15), pediatricians or pediatric neurologists to whom these cases were referred attributed the reactions and their sequelae to pertussis vaccine. They were more likely to do so when there was no record of antecedent abnormality. In 76 cases, including 28 children who reacted to two or more injections, the cause of the reaction was stated to be unknown by consultants (Table 16).

In this connection, I have to acknowledge that many doctors in Britain, the United States, and elsewhere have told me they have never seen a

Table 14. Contraindications to Vaccination in Children with Brain Damage who Receive DTP

Reacting to:	Contraindications					Total
	Family history	Obstetric or perinatal	Other disease	More than one	None	
1 injection only	12	17	15	34	31	109
2 or more	5	12	6	28	37	88
Total	17	29	21	62	68	197

Table 15. Referrals to Hospital of Children with Brain Damage After DTP

Medical Record	Consultants' Opinions as to Cause of Brain Damage					Total
	Genetic	Perinatal	Infection	Pertussis vaccine	Unknown	
Genetic disorder	0	0	0	0	4	4
Perinatal problems	0	3	0	4	4	11
Infection	1	0	0	0	0	1
Other diseases	4	0	2	21	12	39
No antecedent abnormality	5	1	2	78	56	142
Total	10	4	4	103	76	197

severe reaction to triple vaccine, just as several pediatricians have assured me with conviction that they have never seen a child brain-damaged by vaccine. Hospital records and national statistics are so remarkably devoid of any entries that one could say, as I did until 1974, that such incidents reported in the literature are either rare or imaginary. It was only when I was consulted about some fresh incidents in Glasgow that I began to think otherwise (23). Further leads came from scrutiny of hospital records (100,101) and from reports from colleagues, parents and organizations caring for backward children. As a result, the number of cases in my files has grown from 3 in 1974 to over 1,000 today.

All the children in this series are severely brain damaged, with gross mental or physical handicap or both. The evidence is mainly circumstantial and often questionable, but this is true of many medical diagnoses, for instance when we attribute a convulsion occurring during an infection to the pyrexia. So no one can prove that these children were damaged by pertussis vaccine, but neither can anyone prove that they were not. I have excluded from Tables 10–16 any case in which there was confirmation of an alternative diagnosis unless it presented as a recognizable and scheduled contraindication to vaccination. The pattern of reaction and of sequelae is very similar to that in the literature, and I am inclined to think the vaccine is the most

likely explanation of permanent brain damage and disability in most of these cases, which were tabulated either because of clear contraindications to vaccination or because the vaccine evoked a reaction on more than one occasion.

These are all severe cases. It would be out of keeping with other adverse reactions to other substances if the only cases were severe ones. More likely there are numerous cases of lesser damage and lesser disability. The incidence of such disability presumably lies somewhere between the incidence of severe reactions without sequelae, which might be as high as 1:25, and the lesser estimate of permanent brain damage of 1:50,000. The median geometric incidence of such a gradient would be 1:800, which happens to be almost exactly the incidence of the transient cerebral disturbance observed by Linnemann et al. (29) in their adult volunteer series.

No one knows the incidence of lesser degrees of brain damage following vaccination, since contraindications and minor reactions alike can so easily be overlooked in infants. The estimate of 1:800 given above is a shot in the dark—and the darkness is indeed intense in this matter. But even if this estimate is too high, say a tenfold overestimate, the incidence of minor degrees of brain damage could be 1:8000, which means that the number of well children who have suffered mental or physical disturbance as a result of vaccination may have to

Table 16. Consultants' Opinions on Children with Brain Damage After Receiving DTP

No. of Children Reacting to:	Consultant's Opinion as Cause				Cause Unknown	Total
	Genetic	Perinatal	Infection	Pertussis vaccine		
1 injection only	7	2	4	60	48	121
2 injections	3	2	0	25	17	47
3 or more	0	0	0	18	11	29
Total	10	4	4	103	76	197

be counted not in hundreds, as in the United Kingdom, or in thousands as in the rest of the Western world, but in hundreds of thousands all over the world. This is a heavy price to pay for marginal and temporary protection against a disease that seldom nowadays threatens the life or health of well children. The epidemiological evidence is that whooping cough spreads, less from lack of vaccination than from the persistence of too many pockets of residual infection in homes and schools, in areas where living conditions still resemble the slums of earlier decades when whooping cough was a killer disease, or in the primitive living conditions of parts of the Third World, where it still is.

SUMMARY

Pertussis has declined in incidence and severity over the last 50–100 years in all countries where records of comparative incidence are maintained. Epidemic peaks are relatively sharp, consistent with a short period of infectivity and rapid development of natural immunity.

The decline in incidence has followed a rise-and-fall pattern, in which the onset of each successive outbreak depends on the rate of decay of the previous epidemic and growth of new susceptibles during the interepidemic period. The new susceptibles include those without immunity and those who have lost it. The constant presence of numerous proven cases of pertussis in fully vaccinated children proves that vaccines as used at present are incompletely protective.

Deaths and severe complications declined more, by orders of magnitude, before the introduction of mass vaccination than subsequently, which significantly indicates a continuous trend toward a milder form of the disease. The fact that incidence at all ages decreased during a period when the preschool and school population was rising and prior to mass vaccination indicates that herd immunity operates effectively without vaccination.

Pertussis vaccine is intrinsically toxic because potent histamine-sensitizing and other pharmacologically active substances are inseparable from the immunizing components. Adverse reactions are therefore common but usually transient. Severe reactions are uncommon but are followed in a minority of cases by mental or physical defect and other signs of permanent brain damage.

The reported incidence of convulsions after DTP is compared with the probability of a convulsion occurring by chance on the basis of a Poisson distribution in a mathematical model. In children predisposed to convulsions, the probability of a convulsion by coincidence after an injection cannot be excluded. In other children, the probability of a chance convulsion is lower than the frequency reported in some studies of DTP. A second convulsion occurring only after a second or subsequent injection is extremely unlikely to be due to chance.

The incidence of brain damage cannot be stated precisely, because notification procedures are inadequate and because susceptibility appears to be higher in children subject to febrile convulsions or with congenital and other neurological lesions. In the United Kingdom the incidence of permanent brain damage between 1957–1976 is estimated at 1:10,000–1:50,000 children vaccinated. Transient and lesser degrees of brain damage are probably more common.

These risks of vaccination in the United Kingdom are higher for the majority of the child population than the risks of permanent injury or death from whooping cough.

REFERENCES

1. Public Health Laboratory Service. Efficacy of whooping-cough vaccines used in the U.K. before 1968. Br. Med. J. 4:329–333; 1969.
2. Pereira, M.; Candeias, J. A. N. The association of viruses with clinical pertussis. J. Hyg. 69:339–403; 1971.
3. McKeown, T.; Lowe, C. Introduction to social medicine, 2nd edition. Oxford: Blackwell.
4. Whooping cough vaccination: review of the evidence by the Joint Committee on Vaccination and Immunisation. London: Her Majesty's Stationery Office; 1977.
5. Gordon, J. E.; Hood, R. I. Whooping cough and its epidemiological anomalies. Am. J. Med. Sci. 222:333–361; 1951.
6. Kendrick, P. Can whooping cough be eradicated? J. Infect. Dis. 132:707–712; 1975.
7. Medical Research Council. The prevention of whooping cough by vaccination. Br. Med. J. 1:1463–1471; 1951.
8. Medical Research Council. Vaccination against whooping cough. Final report. Br. Med. J. 1:994–1000; 1959.
9. Public Health Laboratory Service. Efficacy of whooping cough vaccines before 1968. Br. Med. J. 1:259–262; 1973.
10. Preston, N. W. Effectiveness of pertussis vaccines. Br. Med. J. 2:11–13; 1965.
11. Preston, N. W.; Stanbridge, T. N. Efficacy of pertussis vaccine: a brighter horizon. Br. Med. J. 3:448–451; 1972.
12. Holt, L. B.; Spasojevic, V. The role of surface antigens in the protective potency of Bordetella pertussis suspensions. J. Med. Microbiol. 1:119–121; 1968.
13. Griffith, A. H. Reactions after pertussis vaccines: a manufacturer's experiences and difficulties since 1964. Br. Med. J. 1:809–815; 1978.
14. W.H.O. Expert Committee on Biological Standardisation. Technical Report Series No. 274. Geneva: W.H.O.; 1964.
15. Manclark, C. R. Current status of pertussis vaccine: an overview. Adv. Appl. Microbiol. 20:1–7; 1976.
16. Department of Health and Social Security. Annual reports of the Chief Medical Officer 1970–1971. London: Her Majesty's Stationery Office; 1971.
17. Office of Population Census and Statistics: Registrar General's weekly returns, 1970–1978. London: Her Majesty's Stationery Office; 1978.
18. Bassili, W. R.; Stewart, G. T. Epidemiological evaluation of immunisation and other factors in the control of whooping cough. Lancet 1:471–474; 1976.
19. Lambert, H. J. Epidemiology of small pertussis outbreak in Kent County, Michigan. Public Health Rep. 80:365–369; 1965.
20. Reynolds, M. R. F. Whooping cough and immunisation in Bristol, 1971. Comm. Med. 36:103–104; 1972.
21. Gostling, J. V. T.; Payne, D. J. H. Whooping cough vaccination. Lancet 2:773; 1974.
22. Miller, C. L.; Fletcher, W. B. Severity of notified whooping cough. Br. Med. J. 1:117–119; 1976.
23. Stewart, G. T. Vaccination against whooping cough: efficacy versus risks. Lancet 1:234–237; 1977.
24. Bennett, N. M. Whooping cough in Melbourne. Med. J. of Aust. 2:481–487; 1973.
25. Rauber, A.; Bruner, B.; Feldman, W.; Nahnias, A.; Elsea, W.; McCoan, J. Pertussis—Georgia. Morbidity Mortality Weekly Report 26:307–308; 1977.
26. Lees, A. W. Atelectasis and bronchiectasis in pertussis. Br. Med. J. 2:1138–1141; 1950.
27. Public Health Laboratory Service. Communicable disease reports. Nos. 1–34. London; 1978.
28. Jenkinson, D. Outbreak of whooping cough in general practice. Br. Med. J. 2:577–578; 1978.
29. Linnemann, C. C.; Ramundo, N.; Perlstein, P. H.; Minton, S. D.; Englender, D. S.; McCormick, J. B.; Hayes, P. S. Use of pertussis vaccine in epidemic involving hospital staff. Lancet 2:540–543; 1975.
30. Wood, M. L. Filtrable toxic substances in broth cultures of H. pertussis. J. Immunol. 39:25–30; 1940.
31. Pittman, M. Bacterial and host factors in the pathogenesis and prevention of whooping cough. Mudd, S., ed. Infectious agents and host reactions, Chapter 11. Philadelphia: W. B. Saunders Co.; 1970.
32. Sen, D. K.; Arora, S.; Gupta, S.; Sanyal, R. K. Studies on adrenergic mechanisms in children immunised with Bordetella pertussis vaccine. J. Allergy Clin. Immunol. 54:25–31; 1972.
33. Demina, A. A.; Semina, A. A.; Trofimora, Y.; Gradieva, A. M. Toxicity of pertussis vaccines. J. Hyg. Epidemiol. Microbiol., Immunol. 19:293; 1975.
34. Maitland, M. B.; Kohn, R.; MacDonald, A. D. The histamine-sensitising properties of H. pertussis. J. Hyg. (Camb.) 53:196–211; 1955.
35. Morse, S. I. Biologically active components and properties of Bordetella pertussis. Adv. Appl. Microbiol. 20:9–24; 1976.
36. Hannik, C. Surveillance of reactions to biologicals. Bull. W.H.O. 55, No. 2:163–165; 1977.
37. Madsen, T. Vaccination against whooping cough. J.A.M.A. 101:187; 1933.
38. Byers, R.; Moll, F. C. Encephalopathies following prophylactic pertussis vaccine. Pediatrics 1:437; 1948.
39. Globus, J. H.; Kohn, J L. Encephalopathy following pertussis vaccine prophylaxis. J.A.M.A. 141:507; 1949.
40. Miller, H. G.; Stanton, J. B. Neurological sequelae of prophylactic inoculation. Q. J. Med. 23:1–7; 1954.
41. Werne, J.; Garrow, I. Fatal anaphylactic shock: occurrence in identical twins following second injection of DPT antigen. J.A.M.A. 131:730–732; 1946.
42. Brody, M.; Sorley, R. G. Neurological complications following administration of pertussis vaccine. N.Y. State J. Med. 47:1016–1019; 1947.
43. Christie, A. B. Infectious diseases: epidemiology and clinical practice. 2nd edition. Edinburgh: Churchill-Livingstone; 1975.
44. Committee on the Safety of Medicines (U.K.) Reports of adverse reactions to pertussis vaccines. London. Department of Health and Social Security; 1964–1976.
45. Toomey, J. A. Reactions to pertussis vaccines. J.A.M.A. 139:448–449; 1949.
46. Grace, H. B. Convulsions and hemiplegia in pertussis prophylaxis. Can. Med. Assoc. J. 63:129–131; 1950.
47. Anderson, I.; Morris, D. Encephalopathy after combined diphtheria-pertussis inoculation. Lancet 1:537–539; 1950.
48. Banks, H. S. Pertussis vaccination and encephalopathy. Br. Med. J. 1:110; 1950.
49. Sutherland, J. M. Encephalopathy following diphtheria-pertussis inoculation. Arch. Dis. Child. 28:149–152; 1953.
50. Cockburn, W. C. Hazards of pertussis vaccination. Br. Med. J. 1:1342–1344; 1959.

51. Hellstrom, B. Central nervous system reaction following pertussis vaccination. Acta Paediatr. Scand. 51:232–234; 1962.

52. Wilson, G. S. Hazards of Immunisation. London: Athlone Press; 1966.

53. Berg, J. M. Neurological complications of pertussis immunisation. Br. Med. J. 2:24–26; 1958.

54. Medical Research Council. Vaccination against whooping cough. Br. Med. J. 1:454–457; 1956.

55. Strom, J. Is universal vaccination against pertussis always justified? Br. Med. J. 2:1184; 1960.

56. Malgren, B.; Vahlquist, B.; Zetterstrom, R. Complications of immunisation. Br. Med. J. 2:1800–1801; 1960.

57. Strom, J. Further experience of reactions especially of a cerebral nature in conjunction with triple vaccination. Br. Med. J. 4:320; 1967.

58. Gainstorp, I. Pediatric neurology. New York: Appleton Century Crofts; 1970.

59. Jeavons, P. M.; Bower, B. D. Infantile spasms. Clinics in Developmental Medicine, No. 15. London: Heinemann; 1964.

60. Jeavons, P. M. Whooping cough immunisation policy. Lancet 2:811; 1957.

61. Dane, D. S.; Haire, M.; Dick, G. Further studies with quadruple vaccines. J. Hyg. (Camb.) 64:475–484; 1966.

62. Lennox, W. G. Epilepsy and related disorders. London: Churchill; 1960.

63. Livingston, S. Comparative management of epilepsy in infancy, childhood and adolescence. Springfield, Ill.: Charles C Thomas; 1972.

64. Department of Health and Social Security. Circulars on vaccination to health authorities. London: Her Majesty's Stationery Office; 1972, 1974, 1976.

65. Miller, C. L.; Pollock, T. M.; Clewer, A. D. E. Whooping cough vaccination: an assessment. Lancet 2:510–513; 1974.

66. Krugman, S.; Ward, R. Infectious diseases of childhood. London, Saint Louis: C. V. Mosby Co.; 1968.

67. Control of Communicable Diseases in Man. Washington, D.C.; Am. Public Health Association; 1965, 1970.

68. World Health Organization. Public Health Report No. 8:47; 1961.

69. World Health Organization. Requirements for pertussis vaccine. Tech. Rep. Series 274:25; 1964.

70. World Health Organization. International conference on application of vaccines. Geneva, World Health Organization; 1971.

71. Griffith, A. H. Reactions after pertussis vaccines: a manufacturer's experiences and difficulties since 1964. Br. Med. J. 1:809–815; 1978.

72. Symposia Series in Immunobiological Standardisation. International Symposium on Pertussis. Basel: Karger; 1970.

73. Mande, R. Pertussis immunisation seminar on immunisation in Africa, 7 Oct. 1971. 125. Centre Internationale de d'Enfance, Paris. 1971.

74. Ehrengut, W. Convulsive reactions after pertussis immunisation. Dtsch. Med. Wochenshr. 99:2273–2275; 1974.

75. Kulenkampff, M.; Schwartzman, S. J.; Wilson, J. Neurological complications of pertussis inoculation. Arch. Dis. Child. 49:46–49; 1974.

76. Dick, G. Reactions to routine immunization of children. Proc. R. Soc. Med. 67:371–372; 1974.

77. Aicardi, G.; Chevrie, J. J. Accidents neurologiques consecutifs a la vaccination contre la coqueluche. Arch. Franc. Ped. 32:309–318; 1975.

78. Gostling, J. V. T.; Payne, D. J. H. Whooping-cough vaccine. Lancet 1:569; 1975.

79. Dolby, Jean M. Whooping-cough vaccine. Lancet 1:804; 1975.

80. Ehrengut, W. Whooping cough vaccination. Lancet 1:370–371; 1978

81. National Childhood Encephalopathy Study. Protocol sent to participating hospitals. London: Dept. of Health and Social Security; 1976.

82. Noah, N. D. Attack rates of notified whooping-cough in immunised and unimmunised children. Br. Med. J. 1:129–129; 1976.

83. Malleson, P. N.; Bennett, J. C. Whooping-cough admissions to a paediatric hospital over 10 years: protective value of immunisation. Lancet 1:237–238; 1977.

84. Stuart-Harris, C. H. This symposium.

85. Volk, V. K.; Gottshall, R. Y.; Anderson, H. D.; Top, F. H. Bumery, W. E.; Serfling, R. E. Public Health Rep. 79:424; 1974.

86. Lennox-Buchthal, M. Febrile convulsions. Amsterdam: Elsevier; 1973.

87 Royal College of General Practitioners. Br. Med. J. 2:416; 1960.

88. Van den Berg, B. J.; Yerushalmy, J. Studies on convulsive disorders in young children. Pediatr. Res. 3: 298–304; 1969.

89. Kurland. L. T. Epilepsia, 4th Series 1:143–161; 1959.

90. Nelson, K. B.; Ellenberg, J. H. Predictors of epilepsy in children who have experienced febrile seizures. N. Engl. J. Med. 295:1029–1033; 1976.

91. Griffith, A. H. Pertussis vaccine and convulsive disorders in childhood. Proc. R. Soc. Med. 67:372–374; 1974.

92. Harker, P. Primary immunisation and febrile convulsions in Oxford 1972-1975. Br. Med. J. 2:491–493; 1977.

93. Perkins, F. T.; Sheffield, F.; Miller, C. L.; Skegg, J. L. The comparison of toxicity of pertussis vaccine in children and mice. International Symposium on Pertussis, Bilthoven 1969. Symp. Series Immunobiol. Standards 13, 14–19. Basel: Karger; 1970.

94. Hilton, M. L.; Burland, W. L. Ibid.150–156.

95. Millichap, J. G. Febrile convulsions. New York: Macmillan; 1968.

96. Wallace, S. Factors predisposing to a complicated initial febrile convulsion. Arch. Dis. Child. 50:943–947; 1975.

97. Kurokawa, T.; Yokota, K.; Takashima, S.; Nambu, Y.; Hanai, T.; Goya, N. Etiology of conversions in neonatal and infantile period. Folia Psychiatr. Neurol. Jap. 30:366–377; 1975.

98. Webber, R.; Craig, J. Socio-economic classification of local authority areas. Studies on medical and population studies No. 35. London: Office of Population Census and Statistics; 1978.

99. Weekly, monthly, and annual Reports of the Registrars-General and Office of Population Census and Statistics. 1900–1978. London and Edinburgh: Her Majesty's Stationery Office; 1978.

100. Scottish hospital in-patient statistics. Edinburgh: Her Majesty's Stationery Office; 1964–1976.

101. Hospital in-patient enquiry. London: Her Majesty's Stationery Office; 1967–1975.

Pertussis Vaccine Experience in the Netherlands
C. A. Hannik and H. Cohen

ABSTRACT

A mortality from whooping cough of about zero during the last 14 years and a low morbidity rate indicate the effectiveness of pertussis vaccination in the Netherlands. In our opinion, the use of a sufficiently potent pertussis vaccine and a vaccination acceptance rate of about 90% of infants are major factors in the achievement of this epidemiologic situation.

Unfortunately, pertussis vaccination may be followed by an adverse reaction. The most common major reactions are shock and convulsions. The incidence after injection with an $AlPO_4$-adsorbed DTP-Polio vaccine with a concentration of the pertussis component per dose of 16 OU is estimated to be 1:2700 vaccinated children. This incidence is not influenced by reducing the pertussis component to 10 OU per dose.

In contrast to shock and convulsions, encephalopathy is observed very rarely. No cases have become known in the last 5 years. Therefore estimation of this incidence in the Netherlands is unreliable.

Mortality from whooping cough in children up to 15 years of age in the Netherlands shows the well known pattern of decay (Fig. 1). Mortality has been notifiable in our country since 1905. A slow but continuous decline occurred from 1905 on, then an increase during the Second World War, followed by a rather sharp reduction in the early fifties.

Physicians generally agree that this reduction was caused mainly by the introduction of chemotherapeutics and antibiotics. Though antibiotic treatment does not influence clinical pertussis, it has an important effect on the rate of secondary bacterial infections and consequently on the mortality rate.
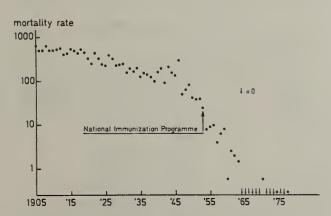
Figure 1. Death rate due to whooping cough per million children up to 15 years of age.

In 1953 immunization against pertussis with DP- or DTP-vaccine, both containing $AlPO_4$ as adjuvant, was started on a national scale in the Netherlands. The first target population was the infants, who received three monthly injections beginning at the age of 3 months. Vaccination and systematic revaccination of toddlers followed gradually. The acceptance rate among infants was 70–75%. The concentration of the pertussis component in the vaccine per dose was equivalent to 20 International Opacity Units, but the potency did not meet the WHO requirements of 4 IU per dose. A DTP-vaccine with a pertussis component concentration of 16 OU per dose and a potency of at least 4 IU per dose was introduced in 1959.

At the end of 1962 a major change was made both in the vaccine and in the vaccination schedule. DTP-vaccine was withdrawn and replaced by DTP-Polio vaccine. The infants then received four DTP-Polio injections instead of three injections with DTP-vaccine. The primary injections were given according to the same schedule used for DTP-vaccinations, at 3, 4, and 5 months of age, but a fourth or booster injection was added at the end of the first year of life, at least 6 months after the third inoculation. Reinforcing doses with the pertussis component at toddler age was stopped gradually in later years.

The use of a quadruple vaccine had an important effect on the acceptance rate. From then on,

over 90% of all newborns received a complete immunization.

Since 1964, a year after the introduction of the DTP-Polio vaccine, death from whooping cough has become rare in the Netherlands.

A reduction of the concentration of the pertussis component from 16 OU to 10 OU per dose, did not affect the mortality rate of about zero.

Notification of morbidity due to whooping cough has been mandatory in the Netherlands since January 1, 1976. As only bacteriologically confirmed cases are accepted on the list of notification, these numbers underestimate the real prevalence of the disease. Personal reports gave the impression that there was more whooping cough in 1977. The actual number of notifications confirms this impression: 1976: 4; 1977: 25; 1978: 1 (up to October 15). The vaccination status of 28 of these children is known; 1 of them was vaccinated completely, and 3 had received one injection.

The best information available is hospital admissions with the clinical diagnosis of whooping cough (Fig. 2). The data demonstrate that whooping cough has become rare in our country. The disease will not disappear completely as long as we have our "black spots," areas where vaccination is refused on religious grounds.

Because the mortality level has been about zero for 14 years and because of the low level of morbidity, mainly among unvaccinated children, we are convinced of the effectiveness of pertussis vaccine, provided a vaccine of sufficient potency is used and vaccination is accepted for at least 90% of infants.

We are aware that vaccine-induced adverse reactions do occur and that the more favorable the epidemiologic situation is, the less acceptable these reactions are, for both the doctors and the public.

Since the introduction of DTP-Polio vaccine in the Netherlands, every case of a possible adverse reaction reported to the Rijks Instituut voor de Volksgezondheid has been analyzed and recorded. The most common major reactions are shock or collapse and convulsions. During a period of 12 years of immunization with the 16 OU DTP-Polio vaccine 53 cases of shock were diagnosed (Table 1). With one exception, the reaction occurred within 6 hours after vaccination, mainly in young babies after the first injection. All children recovered completely without therapy. Recurrences have been observed after further injection with the pertussis component. Because of the close time rela-
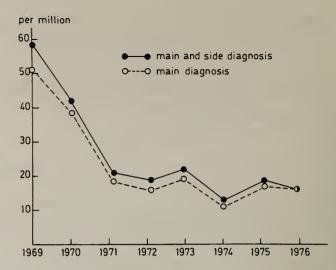
Figure 2. Hospital admissions because of whooping cough per million children up to 15 years of age.

tionship of recurrences to further vaccination and the absence of other etiological factors, we believe the shock is vaccine-induced. Therefore the occurrence of shock is considered to be a contraindication for further injections with DTP-Polio vaccine. In these cases immunization is completed with Diphtheria-Tetanus-Polio vaccine, which is always tolerated without any untoward reaction.

During the same period, 37 cases of convulsions were observed up to 3 days after vaccination. The criteria for the diagnosis "convulsions" are disturbed consciousness and some muscular contractions. Therefore the clinical picture of this group may range from staring and twitching of fingers or mouth to generalized convulsions, sometimes prolonged, sometimes recurrent. Staring and twitching are reported by the mother, whereas the most serious reactions (Table 2) are reported by the clinician in the hospital to which the child has been admitted.

Table 1. Shock After Injection with DTP-Polio Vaccine —16 OU

Age (months)	Number of Cases After Injection				Total number of cases
	No. 1	No. 2	No. 3	No. 4	
2	7	—	—	—	7
3	22	1	—	—	23
4	5	5	1	—	11
5	—	2	5	—	7
6	—	1	—	—	1
11	—	—	—	3	3
15	—	—	—	1	1
Total	34	9	6	4	53

Table 2. Number of Convulsions After Injection with DTP-Polio Vaccine—16 OU

Age (months)	Injection No. 1	No. 2	No. 3	No. 4	Total
2–6	14	6	4	—	24
7–16	3	—	1	9	13
Total	17	6	5	9	37

Children with convulsions after injection with DTP-Polio vaccine show more variation in age and number of injections than the children in the shock group. Also the time interval between injection and onset of symptoms ranges from 30 minutes to 72 hours. Evidently this group is not homogeneous. There are children with febrile convulsions. There are children from families with positive neurological histories who might have had their first convulsion spontaneously. But there still are some children with no other reason for the convulsion than the vaccination.

The incidence of a family history positive for neurologic disorders in this group is rather high (Table 3). About half of the children have a positive history, compared to 19% of the children in the shock group. This is striking, considering the occurrence of convulsions in the near family. About one-third of the neurologic disorder cases have a positive history of convulsions in the near family, compared to 4 of 47 shock cases. Because of these findings, vaccination with the pertussis component is now contraindicated in our country for children with a history of convulsions or epilepsy in the near family or a personal history of convulsions.

Occasionally, convulsions are prolonged, recurrent, and difficult to control. Such convulsions may result in permanent brain damage. It is not important whether these reactions are diagnosed as "status convulsivus" or "encephalopathy"; the outcome is the telling factor.

During the 12 years of immunization with the 16

Table 3. Neurological Family History of Children Reacting with Convulsions or Shock After Injection with DTP-Polio Vaccine—16 OU

Nature	Immediate Family Positive	Immediate Family Negative Parents Positive	Negative	Total
Convulsions	12 (37.5%)	5 (15.6%)	15 (46.9%)	32
Shock	4 (7.8%)	6 (11.8%)	41 (80.4%)	51

OU DTP-Polio vaccine we observed five such cases. One recovered completely, one still has slight sequelae from a hemiparesis, and three babies died. In addition, two children have been observed who showed convulsions and changes in consciousness 72 hours after a DTP-Polio vaccination. They deteriorated quickly and both suffer from permanent cerebral damage. Serological examination demonstrated a more than fourfold rise in *Herpes simplex* complement fixing antibody titer. The cerebrospinal fluid of both children showed some pleocytosis.

Whenever possible, virologic and serologic examination should be done to diagnose coinciding virus infections. A positive result does not exonerate the pertussis vaccination as the only cause of the illness, but a primary infection, which alone could cause the same symptoms, might be triggered when combined with an inoculation.

When we learned the nature of most common reactions to DTP-Polio vaccination, we set up a prospective study on the incidence in several baby clinics in the province of South Holland.

The frequency of reporting of major reactions after pertussis vaccination depends on attention and thoroughness in searching for them. In the Netherlands one batch of DTP-Polio vaccine is distributed all over the country every 3 months. Reports of adverse reactions should therefore be scattered over the country, but some provinces have never reported a reaction. The province of South Holland shows a higher incidence of shock and convulsions than the rest of the Netherlands. The baby clinics in the Hague, one of the two big cities in South Holland, have always been very reliable in the control and reporting of adverse reactions. The routine procedure in the baby clinics is that from the first visit, the mother is invited to contact the clinic if she is worried, and each mother is questioned systematically about a possible reaction after a previous vaccination before a further injection is given. As soon as the doctor of the baby clinic considers the possibility of an adverse reaction, an interview is arranged with the mother to make a complete record. In this way, 46,000 infants in the Hague have participated in the study so far.

The incidences of shock and convulsions observed up to 3 days after injection of the 16 OU DTP-Polio vaccine are about the same: 1 in about 2,700 vaccinated children (Table 4).

The occurrence of seven cases of "encephalopathy" after injection of the 16 OU DTP-Polio vac-

Table 4. Incidence of Shock and Convulsions After Injection with DTP-Polio Vaccine—16 OU per 10,000 Vaccinated Children

Period of Observation	Estimated Number Vaccinated Children	Incidence of Reactions [a] Shock	Convulsions
	South Holland		
1968–1975	292,293	0.6 (17)	0.3 (·9)
	The Hague		
1969–1975	35,284	3.7 (13)	3.7 (13)

[a]Total number of reactions given in parentheses

cine, observed in the Netherlands during a period of 12 years, including the two cases with a coinciding primary infection with *Herpes simplex* virus, means an incidence of about 1 in 400,000 vaccinated children. We assume this complication is not much underreported. Two of these cases were from the Hague.

Impressed by the frequency of major reactions such as shock and convulsions, we considered measures to reduce the number of adverse reactions. A theoretical possibility was reduction of the concentration of *Bordetella pertussis* organisms in the vaccine, particularly because the potency of the pertussis component was considerably higher than the minimum required value of 4 IU per dose. To a certain extent this idea was not reasonable, as some
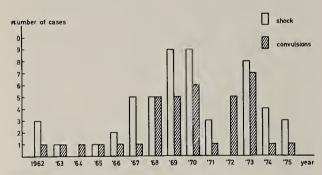
Figure 3. Periodicity of shock and convulsions after injection with DTP-Polio vaccine—16 OU.

children who had one major reaction showed a recurrence after further injections with half a dose or even with one-tenth of a dose of 16 OU DTP-Polio vaccine. Nevertheless, it was decided to reduce the concentration of 16 OU per dose to 10 OU while maintaining the minimal potency of 4 IU.

Over the years a certain periodicity in reporting has been noticed. It is not exactly a clustering of reactions but a variation (Fig. 3) in which there are years with some cases and years with more than 10 cases. We think now that this is due to batch variation in vaccine production.

At first, reduction of the pertussis component appeared to have solved the problem of adverse reactions, since no major reactions were reported during a period of 1.5 years following that modification. Such a long period without one major reaction had never been observed before. From then on, however, adverse reports were received. Table 5 shows that the incidence of shock and convulsions has not been changed by reducing the concentration of the pertussis component. The period of observation is rather short and 1975 has been excluded, but one gets the impression that reducing the concentration of the pertussis component to an equivalent per dose of 10 OU does not solve the problem of adverse reactions.

There is just one hopeful observation, which we make with a certain reserve: since the introduction of the 10 OU DTP-Polio vaccine, not a single case of encephalopathy has been reported in the country.

Table 5. Incidence of Shock and Convulsions After Injection with DTP-Polio Vaccine—10 OU per 10,000 Vaccinated Children, 1976–August 1978

Estimated Number Vaccinated Children		Incidence [a] Shock	Convulsions
South Holland	73,000	0.14 (1)	0.27 (2)
The Hague	11,000	3.6 (4)	3.6 (4)

[a]Total number of reactions given in parentheses

Pertussis in Developing Countries: Possibilities and Problems of Control through Immunization

R. Cook

We should begin by reminding ourselves that the problem of pertussis in the developing countries of Africa, Asia, and Latin America is many times more serious than it is in Europe and North America. The severity of the problem has three elements: 1) a lower median age of incidence, which in Morley's survey (1) from 17 developing countries was mostly in the last part of the second or in the third year of life (Table 1); 2) an association with protein-energy malnutrition; 3) a higher case-fatality rate.

The combination of these three factors is responsible for a much higher mortality than in the industrialized countries, as can be seen for example in successive issues of "Health Conditions in the Americas" (2–4); higher in absolute numbers, higher per hundred thousand population, and higher in rank order as a cause of death in childhood. In fact it would be no exaggeration to say that more than 95% of pertussis deaths occur in the developing countries. We can find plenty of evidence to support this assertion in small retrospective or even prospective surveys of mortality and in accounts of admissions and deaths in pediatric wards in the tropics. However, even more than with the other childhood diseases preventable by immunization, we are soon at a loss if we want reliable or even remotely indicative data on cases and deaths nationwide from official sources. Moreover, if we want to look in greater detail at any one of the factors mentioned, whether it be lower age of incidence, association with malnutrition or case fatality rate, we will be surprised by the scarcity of published studies on any of these three aspects. In fact, Morley's classic paper (1) still remains, 12 years later, pretty well alone as a review of that scope. If pertussis causes only 2% of infant and preschool deaths in the developing countries, however, that would be over a quarter million a year, and for a disease with a death toll of this magnitude it is remarkably underinvestigated in the countries where it principally occurs. Of course 2% is a guess. It was 0.85% in the 13 Latin American/Caribbean projects in the Inter-American Investi-

Table 1. Median Age in Years of Whooping Cough Notification.[a] Figures from 17 Countries (1963–64)

Country	No. of Cases Notified	Median Age	Country	No. of Cases Notified	Median Age
Basutoland	115	2.2	Morocco	89	2.9
Burma	198	3.0	Nigeria (E)	345(12)	1.8
Congo	117(2)	1.6	Nigeria (N)	75	2.9
Egypt	449	3.0	Nigeria (W)	575(3)	2.4
Ghana	349(3)	2.2	Malawi	121	1.8
India	250(4)	2.7	Sierra Leone	231(3)	1.4
Indonesia	131	3.1	S. Africa	472(6)	1.7
Jordan	942(2)	2.0	S. Rhodesia	440(7)	2.0
Kenya	277	2.3	Tanzania	669(7)	3.0
Liberia	96	2.2	Uganda	362(4)	1.9
			Zambia	172(4)	2.8
(In countries where records were supplied by more than one doctor the number of doctors is given in parentheses.)					
Aberdeen (1890–1900)	15,094	3.2	Massachusetts (1918)	7,765	4.6
England and Wales (1945)	92,266	4.4	Massachusetts (1945)	7,445	5.2

[a]From Morley et al. (1)

gation of Child Mortality (5), 1.36% in the Afghan prospective study (6), and about 2.75% if one divides the average number of deaths from whooping cough 1969–1972 from the Latin American and Caribbean countries by the total number of deaths among children under 5 years of age.

The major difficulties of investigating pertussis in developing countries include not only the universal difficulty of diagnosis in the infant who may have the disease without exhibiting the characteristic whoop, but also the scarcity of laboratory facilities for the difficult bacteriological confirmation and the fact that the great majority of children who die in the developing countries never see a doctor or a paramedical worker even in their last and fatal illness. On the other hand, in many countries the mothers themselves recognize whooping cough and there is often a vernacular word or descriptive phrase for the disease.

The cost of three doses of triple vaccine purchased by or through UNICEF has come down as of 1977 to 5.1 cents U.S. Even if we multiply the cost by 5 for storage, transportation, and other costs of administering vaccine, it still seems a very good public health bargain.

A major new program of WHO and its member countries has been initiated recently to make pertussis vaccine in the form of triple vaccine, along with BCG, polio, and measles vaccines, much more widely available to the children of developing countries. This Expanded Programme of Immunisation (EPI), as it is called, aims to help the developing countries overcome the major constraints and obstacles standing in the way of effective immunization against these six diseases. These obstacles are:

1. Lack of vaccines and the means to administer them;
2. Lack of enough storage facilities of adequate quality ("cold chain");
3. Lack of rural health facilities and of transportation to reach rural populations;
4. Lack of management capability in the health services;
5. Lack of epidemiologic data and of trained manpower to collect and analyze such data;
6. Lack of public awareness about immunization.

An up-to-date description of the Expanded Programme of Immunisation is given in the WHO document A31/21, which is appended to this report.

We referred earlier to pertussis vaccine in the developing countries as "a good public health bar-

gain." Just how good a bargain it is depends on the answers to certain crucial questions. How effective is pertussis vaccine in preventing pertussis? Can it really be delivered at an appropriate age to a significant proportion of the child population at risk in the typical conditions of a developing country? Has anybody done it? With what effect? What did it cost?

The remainder of this paper attempts to answer these questions with a consideration of hitherto unpublished data from a program in Uganda 1965–1970, which has become of wider interest with the development of EIP.[1]

In the Ankole province of southwest Uganda, an area of approximately 6,000 square miles and close to one million population by 1970, a child health program was carried out from 1965 to 1972 which included comprehensive immunization of children aged 0–4 years (7). There were about 42,000 births a year and the average number of children under 5 years of age was about 176,000. A three visit schedule included three doses of DTP and TOPV plus BCG and smallpox vaccination for all and measles vaccine for children aged 9 to 35 months (Table 2). The immunizations were delivered to the children by a combination of 20 health centers and three mobile teams.

In March 1967, house-to-house random sample survey for BCG scars and completed immunization cards showed 63.3% of children under 5 had completed the three visit schedule, 8.7% two visits only, 5.7% one visit, and 21.3% no visit. In 1967 61.4% had visible BCG scars, which confirms the above; this figure 2 years later in a similar survey was

Table 2. Schedule Used in Ankole Immunization Program, 1965–1970

Visit 1	Interval	Visit 2	Interval	Visit 3
BCG		Measles		
Smallpox	4–8	DTP2	4	
DTP1	weeks	TOPV2	weeks	DTP3
TOPV1				TOPV3

All vaccines were given to any child under 5 years of age not previously immunized, except measles vaccine, which was restricted to children 9–35 months of age.

62.0%. These house-to-house survey figures corresponded closely with the coverage figures obtained from records of numbers of children immunized over estimated population.

Only UNICEF-provided, and therefore WHO-approved, triple vaccine was used. The dropout rate over the period May 1965–December 1970 was such that between 49.6% (fixed health centers) and 52.7% (mobile teams) of those who began a three visit course completed it (8).

The cost was 82 cents U.S. per fully immunized child. This does not take account of those who benefited from one or two visits (BCG, smallpox, measles vaccines) and also ignores the fact that about 20% of the cost went back to the government in tax. Also the vaccines were in fact donated, but are included in the costing as if they were purchased. Thus the actual cost to the government would have been considerably less. Nevertheless, if we stay with the 82 cents figure it closely matches the cost of a later program in 1974–1976 in Kenya of $1.73 per fully immunized child, allowing for inflation between 1967 and 1975 (9).

It is the question of effectiveness that raises much of the difficulty. When one hears such divergent views as these of Stewart (10) and Preston (11) in a medically sophisticated country like the United Kingdom, one realizes that any data from a program in a rural area of a developing country are bound to be challenged, and we must leave the significance of such data to the judgment of this audience.

Table 3 shows the decline in the percentage of admissions at the district hospital attributable to whooping cough from the period November 1963 to June 1966 (the mass immunization began in September 1965) compared with the period July 1967 to June 1968 (the initial round of immunization was completed September 1967).

There were 10 government rural health units in Ankole making quarterly morbidity returns and continuing to do so up to 1971. The numbers of pertussis cases reported quarterly are represented in Figure 1. The mass campaign was completed in September 1967, and it can be seen that the level of pertussis notifications in 1967 and 1968 was much lower than in the previous interepidemic season, January 1963 to June 1964. It rose again in an epidemic period 1969 to 1970 but not nearly to the same heights as before, and it declined again to levels lower than the 1963–1964 interepidemic period. Moreover, in the neighboring districts of Masaka (east of Ankole) and Kegezi (west of Ankole), with populations of 640,000 and 648,000 respectively, where little immunization had been carried out, the notifications in the epidemic year of 1970 were over 2,000 in each case compared with less than half that figure in the much larger district of Ankole.

Table 4 illustrates the level of hospital admissions due to pertussis in the hospitals of the districts east and north (Toro) of Ankole compared with those in Ankole district hospital itself.

However, there are pitfalls in all these data. Declines in morbidity and mortality after epidemics that immediately preceded immunization campaigns can be mistaken for beneficial effects of the immunizations. Moreover, in a case like this, where one is relying on diagnosis by doctors and medical assistants, the knowledge that an immunization campaign with high coverage has been carried out in the district could tend to make these health workers reluctant to make the diagnosis of whooping cough, in the belief that immunization is effec-

Table 3. Admissions and Deaths from the Immunizable Diseases as a Percentage of All Admissions and Deaths, Mabarara Hospital, Ankole. Children 0–6 Years[a]

	Admissions		Deaths	
	Nov. 63–June 66	July 67–June 68	Nov. 63–June 66	July 67–June 68
Total Numbers	3,703	1,469	282	147
Measles and postmeasles pneumonia	15.3%	18.2%	8.5%	7.5%
Pertussis	3.8%	0.8%	4.3%	0.7%
Tuberculosis (all forms)	4.1%	1.8%	7.8%	5.4%
Tetanus	0.3%	0.1%	2.8%	0.7%
Acute polio	0.4%	0	0.7%	0

[a] From Moffat (8)
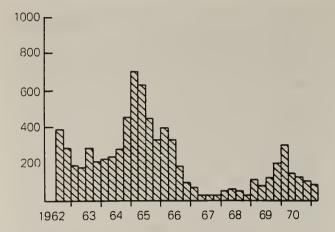
Figure 1. Pertussis quarterly returns, ten health units in Ankole.

tive and the diagnosis therefore less likely to be correct.

In summary, I tentatively answer the questions raised earlier:

—Pertussis immunization in the form of three shots of DTP vaccine *can* be delivered to a significant proportion of children in typical developing country conditions, but it remains to be seen whether it can be done by typical government health services.

—Comprehensive polyimmunization should cost about $2.00 at current prices per completely immunized child. There are pluses and minuses for mobile and fixed center operations and for a mass campaign with wide age range compared with maintenance coverage of annual cohorts of infants.

—Pertussis immunization almost certainly is not in the same class of effectiveness as measles vaccine given at the appropriate age, or tetanus or diphtheria toxoid, or TOPV given often enough. If given to a substantial proportion of vulnerable children (say 60%) it very probably will reduce the level of the problem of pertussis by as much as 50% if our experience in Uganda is a guide.

—Finally, those who believe that pertussis immunization is justified in industrialized countries should believe it ten times more so in the developing world, since the problem is that much greater there. Those who believe that pertussis vaccination is not justified in industrialized countries would surely have scruples about recommending its exclusion from a polyimmunization schedule in developing countries in view of the considerations presented earlier.

REFERENCES

1. Morley, D.; Woodland, M.; Martin, W. J. Whooping cough in Nigerian children. Trop. Geogr. Med. 18:169–182; 1966.
2. P.A.H.O./W.H.O. Health conditions in the Americas, 1961–1964. P.A.H.O. Scientific Publication No. 138, Washington, D.C.; 1966; 66.
3. P.A.H.O./W.H.O. Health conditions in the Americas, 1965–1968. P.A.H.O. Scientific Publication No. 207. Washington, D.C.; 1970; 123–124.
4. P.A.H.O./W.H.O. Health conditions in the Americas 1969–1972. P.A.H.O. Scientific Publication No. 287. Washington, D.C.; 1974; 50–51, 97–118 and 130.
5. Puffer, R. R.; Serrano, C. V. Patterns of mortality in childhood. P.A.H.O. Scientific Publication No. 262. Washington, D.C.; 1973; 61, 155.
6. W.H.O. Infant and early childhood mortality in relation to fertility patterns: report on an ad-hoc survey in greater Kabul Afghanistan 1972–1975. Ministry of Public Health, Kabul and W.H.O. eastern Mediterranean Regional Office, Alexandria. Document EM/ST/120; 1978; 172–173.
7. Cook, R. Child health in Ankole, Uganda. A programme of immunization and health education and its evaluation. University of Oxford; 1969. Dissertation.
8. Moffat, W. M. U. Mobile young child clinics in rural Uganda. University of Edinburgh; 1976. Dissertation.
9. Mahieu, J. M., Summary of a study of the operational feasibility, coverage and costs of maintenance immunizations in children by district mobile teams in Kenya. W.H.O. Monographed Document EPI/WG/76.8.
10. Bassili, W. R.; Stewart, G. T., Epidemiological evaluation of immunization and other factors in the control of whooping cough. Lancet 1:471–474; 1976.
11. Preston, N. W. Protection by pertussis vaccine. Little cause for concern. Lancet 1:1065–1067; 1976.

Table 4. Admission for Whooping Cough, Three District Hospitals in Uganda, 1964–1969[a]

	July 64–June 66 (Average of 2 years)	July 66–June 67	July 67–June 68	July 68–June 69
Ankole	69	8	11	28
Masaka	110	128	211	86
Toro	225	171	181	224

[a] From Moffat (8)

WORLD HEALTH ORGANIZATION

ORGANISATION MONDIALE DE LA SANTÉ

THIRTY-FIRST WORLD HEALTH ASSEMBLY

Provisional agenda item 2.6.10

EXPANDED PROGRAMME ON IMMUNIZATION

Progress report by the Director-General

> This report is presented to the Health Assembly in response to resolution WHA30.53. Progress in the planning and implementation of this programme is described, training activities and developments in the cold chain being emphasized as essential elements for those countries planning to expand their immunization coverage. The growing number of developing countries now actively participating in the programme is noted, as are their requirements for increased support from outside sources in order to ensure acceptable coverage on a continuing basis.

CONTENTS

1. PROGRAMME OBJECTIVES

The Expanded Programme on Immunization (EPI) has its basis in resolution WHA27.57, adopted by the World Health Assembly in May 1974. General programme policies were approved in resolution WHA30.53, adopted in May 1977. The programme's long-term objectives are to:

- reduce morbidity and mortality from diphtheria, pertussis, tetanus, measles, poliomyelitis and tuberculosis by providing immunization against these diseases for every child in the world by 1990 (other selected diseases may be included when and where applicable);

- promote countries' self-reliance in the delivery of immunization services within the context of comprehensive health services;

R 778

- promote regional self-reliance in matters of vaccine quality control and vaccine production.

In the medium term, the programme seeks to:

- develop appropriate immunization plans at regional and country level;

- develop strategies for training national and international staff in the variety of disciplines required for successful programme planning and implementation;

- attract the investment of external funds from bilateral and multilateral sources to support programme activities at global, regional and country levels;

- develop country, regional and global management information systems which assess progress in achieving the programme's objectives accurately and continuously;

- increase the efficiency and effectiveness of the strategies recommended to reduce morbidity and mortality from the target diseases;

- improve the safety, potency, stability, ease of administration, and efficiency of production of all vaccines in which the programme has an interest;

- improve and develop the equipment required for programme implementation so as to increase its suitability for programme purposes, to decrease its cost and, where applicable, to facilitate its manufacture within developing countries;

- improve all aspects of programme management by applying the knowledge obtained from management information-evaluation systems and from research to promote the use of the most efficient and effective disease control techniques appropriate to each country;

- develop global and regional strategies to meet programme requirements for vaccine quality control, production, and distribution;

- promote immunization delivery as a component of comprehensive health services provided to the entire population through cooperation with national governments and in collaboration with other WHO programmes.

Based on the policies approved by the Thirtieth World Health Assembly, the above long-and medium-term objectives dictate the present programme strategy of initially developing managerial competence at senior and middle levels to serve as a foundation for solid and enduring programme implementation.

2. PROGRESS TO DATE

Progress up to May 1977 was summarized in previous reports submitted to the Health Assembly (documents A28/WP/5, A29/16 and A30/13).

2.1 Planning

2.1.1 Global

Regional and national authorities have been made a part of the global planning process through on-site visits and through periodic meetings involving global, regional and country staff. The creation of the EPI Global Advisory Group will further strengthen this collaboration to plan and coordinate the global programme, with emphasis on:

- operational implementation in regions and countries;

- development of prototype training curricula and educational materials;

- development of alternative operational and evaluation strategies;

- development and transfer of appropriate technologies;

- establishment of two-way information systems to obtain global data on the target diseases and the vaccine requirements, and to dispense data to support country decision-making;

- attraction and coordination of extrabudgetary resources.

Following the Thirtieth World Health Assembly, and later in conjunction with the WHO interregional Training Course on Planning and Management for EPI held in Kuala Lumpur, national advisers and WHO staff with regional EPI responsibilities met to coordinate regional and national planning. These discussions proved useful in developing interregional plans of common interest. National participation has focused attention on "consumer" problems with implications beyond the specific countries involved. Such consultations will again be held in 1978, after the Thirty-first World Health Assembly and in conjunction with the first meeting of the EPI Global Advisory Group.

As recommended by an informal EPI consultative group meeting in October 1977, an EPI Global Advisory Group is being appointed which will:

- advise the WHO Secretariat with respect to programme priorities over the short, medium and long term;

- promote the exchange of information concerning programme strategies and tactics among participants at country, regional and global levels; and

- promote the understanding of, and support for, programme goals among technical and political leaders.

The first meeting of the Group is scheduled for November 1978 and a report of its discussions and recommendations will be made available.

2.1.2 Regional

During the past year all the regional offices have established active EPI focal points, and in three of them personnel have been assigned exclusively to deal with EPI coordination and cooperation with the countries in the Region. In three regional offices this coordination is being carried out within the broader framework of communicable disease or maternal and child health activities.

The present status of country planning and implementation, towards which all global and regional efforts are directed, is indicated below:

Region	Number of Member States in the Region	Number of Member States committed to and cooperating with WHO in the expansion of their immunization services [1]
Africa	42	11
Americas	29	2
South-East Asia	10	9
European	34	-
Eastern Mediterranean	22	15
Western Pacific	15	5

The above obviously does not present the complete EPI picture in that in each region noted, particularly in the European Region and the Region of the Americas, there are countries whose immunization services are fully supported by the country itself, and these, as well as those countries actively cooperating with WHO, must certainly be considered as working toward the common global objectives of the programme.

The Thirtieth World Health Assembly, in resolution WHA30.54, drew attention to the importance of the promotion of regional and national self-reliance in vaccine production. During the past year the following developments are to be noted:

- WHO, through its Biologicals unit, has written and distributed draft manuals on the production and quality control of diphtheria, tetanus and pertussis vaccines, and has organized a course to teach the techniques of measles and poliovirus titrations. These activities were supported by UNDP, as was the organization of two seminars covering the establishment and maintenance of quality control laboratories held in 1977 (Geneva and Mexico City); and consultant visits concerned with quality control and production were made to India, Indonesia, Iraq, Jordan, Philippines, Sudan and Tunisia.

- In the Americas, assistance is being given to government laboratories in obtaining satisfactory production strains, and improving their vaccine production and control procedures. Plans are being made for two vaccine reference laboratories, which will function as training centres and will provide testing services.

- In South-East Asia, following discussions in the Regional Committee, a special meeting is being called in 1978 to discuss the regional production of vaccines for EPI.

- The Regional Office for the Western Pacific has conducted a series of visits to establish control laboratories which will handle vaccine re-testing for those countries of the Region where it is not feasible to establish full-scale quality control facilities.

[1] Africa: Gambia, Ghana, Ivory Coast, Kenya, Liberia, Mozambique, Nigeria, Senegal, Sierra Leone, United Republic of Tanzania, Zambia.

Americas: Ecuador, Guatemala.

South-East Asia: Bangladesh, Burma, India, Indonesia, Maldives, Mongolia, Nepal, Sri Lanka, Thailand.

Eastern Mediterranean: Afghanistan, Democratic Yemen, Egypt, Iraq, Jordan, Lebanon, Oman, Pakistan, Qatar, Saudi Arabia, Somalia, Sudan, Syrian Arab Republic, Tunisia, Yemen.

Western Pacific: Lao People's Democratic Republic, Malaysia, Papua New Guinea, Philippines, Tonga.

..2   Training

EPI training is a major responsibility at both the global and regional levels.   Although training for national staff is a country responsibility that must be adapted to the particular conditions and strategies in each case, WHO headquarters and regional offices have been active in developing training courses and training materials on an interregional and inter-country basis, emphasizing those management and technical areas that are broadly relevant to immunisation programmes.   In addition to avoiding duplication of training effort in many cases, these intercountry and interregional activities have the advantage of broadening the perspectives of both national and international staff through a sharing of problems and experience arising from individual country programmes.

Up to 1977 six EPI seminars had been held, in Africa, the Americas and South-East Asia, which proved invaluable as forums for raising the consciousness concerning immunization and exposing the problems being faced by countries preparing to embark on expanded immunisation programmes.   The training activities described below are an effort to resolve specific common problems in programme implementation.

2.2.1   Training course on planning and management for EPI

In collaboration with the Center for Disease Control of the United States Public Health Service, an innovative training course has been developed covering initial planning and organisation management of a country immunisation programme, based on hypothetical data that approximate to actual conditions in many developing countries.   Participants (including both national and international staff) work individually and in small groups, assisted by a "course manager", and successively solve the problems or consider the alternatives presented in a series of "modules" covering the logical development of an immunisation programme:

(1)   Problem identification and quantification

(2)   Priorities

(3)   Objectives

(4)   Work systems

(5)   Evaluation

Although the course has been designed as an individual exercise, flexibility has been built in to provide for small group discussions on common problems and lecture/discussions for the entire group on specific technical issues of general interest.   The course aims at "total immersion" in that for the two-week period course sessions are arranged on the premises where the participants are housed.

The first such course was held in Kuala Lumpur in October and November 1977, with 50 WHO and national participants from five of the regions and from headquarters.   Based on this experience, and on the positive response of the participants, the course is now being revised and the material translated into French and Spanish.   Future courses based on this material are now being planned in all the regions for 1978 and 1979 so that, by the end of this period, senior health officials with specific responsibilities for immunisation from every country involved will have been exposed to this practical exercise and given the opportunity to share their experiences with national and international colleagues.

2.2.2   Middle management training at the national level

There is an increasing demand from countries now in the pre-operational phase of their immunization activities for training of middle-level supervisory personnel involved in the day-to-day management of these activities.   Prototype curricula are now being developed for the training of district supervisors, health centre officers, and vaccination team leaders covering the basic operational aspects of the programme.   Each course will require to be adapted from the prototype version to meet the particular training needs within each country, and WHO collaboration will be focused on curriculum development with the national officers responsible, and on limited teaching and financial assistance when required.

2.2.3   Training in cold chain management

The problems connected with the cold chain are common to every country, and thus, as noted below, research and development in this area is a priority for the programme.   Current operations, however, cannot be suspended pending technological improvements and innovations, and there is an urgent need to improve the handling of vaccines, using the equipment currently available.   In cooperation with Appropriate Health Resources and Technologies Action Group Ltd, and the Regional Office for the Americas, a training course for national technical officers will be given in Latin America and, after revision and translation, will be available to all regions in the second half of 1978.   The course will generally be given on an inter-country basis, a primary aim being that those trained will have the necessary competence to conduct subsequent training and supervision at the national level.

In addition to the above course, training aid materials for cold chain management are being prepared, in the form of:

- a twenty-minute colour film, shot in Ghana, illustrating correct procedures for vaccine handling, from arrival at the airport to immunisation at the periphery,

- a set of 50 colour slides covering vaccine handling and basic maintenance of refrigerators and freezers.   Two accompanying texts will be included - one for vaccination team members, storekeepers and drivers, the other for senior managers.

2.2.4   EPI field manual

The above manual is now available in English, French and Spanish, and has been widely distributed.   It is composed of five books, dealing with:

(1)   Programme design

(2)   Programme management

(3)   Vaccine handling

(4)   Health education

(5)   Programme evaluation

They have been designed as a reference work and guide for national planners responsible for planning, training and management.   The manual has provided the technical foundation for the training courses described under sections 2.2.1 to 2.2.3 above, and is being transformed and translated into national immunisation manuals in several countries.

2.3   Research and development

2.3.1   Cold chain

Emphasis has been given to the improvement of equipment used in the cold chain; to the improvement of management systems in which that equipment is to be used; and, as noted above, to the training of programme personnel in the use of appropriate equipment in an appropriate management system.

Many cold chain products now in use in developing countries are manufactured for use in the developed countries, where cooler ambient temperatures, assured sources of power, and good maintenance and repair facilities are generally found.   Market forces have not provided a strong stimulus for producing materials specifically designed for the more demanding situations found in many developing areas, with the consequence that much of the burden of adaptation and development of suitable equipment has fallen on WHO, UNICEF, and individual collaborating donor countries.

Notable progress has been made in the development of cold boxes for the transport of vaccines in vehicles, and in the development of portable vaccine containers.   The cold box will keep vaccines between 0°C and +8°C for one week in field conditions where the ambient temperature is +34°C, and under the same conditions the portable vaccine container will keep the vaccine at the proper temperature for two days.   Detailed instructions for the manufacture of cold boxes have been widely disseminated, while the portable containers are commercially available.

The development of refrigerators and freezers for use in areas without electricity or in areas with intermittent or poor electricity supplies poses more complicated problems, and an additional two to three years will be required before significant improvement can be expected.   In conjunction with UNICEF, which is the major purchaser of this type of equipment for use in EPI programmes, and supported by contributions to the Voluntary Fund for Health Promotion, contractual agreements have been made with independent testing laboratories and scientific institutes to carry out tests on currently available refrigerators and modified freezers.   These tests will determine internal temperatures, the effects of door-opening, temperature-rise characteristics, and effects of voltage reduction.   In addition to influencing current purchasing policies, these tests will indicate to countries engaged in EPI activities how best to utilise the equipment currently available to them, and how modifications can be made in the field and at the time of manufacture to ensure maximum protection of the vaccine.   Similar ranges of tests are being carried out for cold boxes, vaccine carriers, cold packs, and dial thermometers.

Consultancies sponsored by WHO and UNICEF have been undertaken in 11 countries during 1977 with the object of improving cold chain management systems.   The work has been mainly focused on vaccine distribution methods within the countries concerned, equipment maintenance, and evaluation and monitoring of the cold chain.   In most of the countries visited the distribution systems being employed jeopardized the potency of the vaccines, and solutions are being sought through training of personnel and through the introduction of simple systems of recording and reporting.   A universal need for better equipment maintenance, particularly day-to-day preventive maintenance, has been observed.   Solutions are being sought through training and through the provision of adequate supplies of spare parts.   The use of UNICEF's organization for transport and maintenance to support ministries of health in carrying out EPI training and equipment maintenance is being pursued.

The application of enzyme indicators to EPI cold chain management is being evaluated.   These indicators change colour after being exposed for a given period at a given temperature, the period shortening as the temperature rises.   They appear to be inexpensive and reliable enough to be attached to shipments of vaccines, where their change of colour would warn storekeepers and users either to use the vaccines immediately, to test them, or to discard them.   Results of studies being conducted in Ghana on the use of these indicators in the field are expected by the end of 1978.

2.3.2   Vaccines

Work continues on the development of more stable, more potent and less reactogenic vaccines for EPI:

Measles.   Success has been reported in producing a more stable freeze-dried measles vaccine, limited quantities of which are expected to be on the market during 1978.   WHO and the State Institute for Public Health, Bilthoven (Netherlands), are supporting studies to further characterize the stabilizing effect of various alterations in the reconstituting fluid for the freeze-dried vaccine, with encouraging preliminary results.   Studies to increase the stability of liquid (e.g., unfreeze-dried) vaccine are continuing under the sponsorship of WHO, UNDP and the London School of Hygiene.

Poliomyelitis.   With WHO and UNDP collaboration, studies of suspending media and materials for containers used for the distribution of oral poliomyelitis vaccines are being pursued by the National Institute of Biological Standards and Control, London, and the National Institute of Hygiene, Budapest.

Diphtheria/pertussis/tetanus.   WHO and UNDP are collaborating with the Institute for Serobacteriological Production and Research, Budapest, the Mechnikov Research Institute for Vaccine and Sera, Moscow, and the Institute of Immunology, Zagreb (Yugoslavia), to improve the stability of the pertussis component of DPT vaccines through the use of freeze-dried vaccines (USSR and Hungary) and through the better characterisation of the stability of pertussis vaccines made from extracts of single strains of Bordetella pertussis (Yugoslavia).   WHO has organized collaborative studies to characterize the reactogenicity and toxicity of different DPT vaccines in Czechoslovakia, the Federal Republic of Germany, the Netherlands, Poland, Romania, Sweden, the United Kingdom, the United States of America, and the USSR.   In addition, encouraging work on preparing less reactogenic pertussis vaccines, using subcomponents of the organism rather than whole cell extracts, has been reported from Japan, Sweden and the USSR.   In October 1977, with UNDP assistance, WHO convened an informal working group on the production and testing of pertussis vaccines, attended by 25 participants in addition to members of the WHO Secretariat.   Current progress and problems in the field were reviewed, and a collaborative plan of action for future research work was agreed to.   Further meetings are planned for 1978.   In addition, the WHO requirements for biological substances pertaining to diphtheria toxoid, tetanus toxoid, and pertussis vaccine (all dating from 1964) have been updated to reflect advances in technology, bringing them into full accord with the WHO production and quality control manuals drafted in 1977.

2.4   Immunization status

At the present time, reliable data on the current status of immunization programmes against the EPI target diseases are available from few Member States.   Despite this, the data which are available have been summarized since they do permit some broad generalizations to be made, and are of historical interest as a baseline against which the improvements which are currently occurring in country health information systems and in actual immunization coverage can be measured.

Using criteria which vary somewhat between regions, 42 developing countries, in which some 57 million children are born annually, have been identified as expanding their immunization programmes in active collaboration with WHO.   Not all of these countries have reported to WHO the number of immunizations provided against the EPI target diseases, and some of those which did report may not have included all immunizations actually given (those administered in the private and voluntary sectors being sometimes difficult to obtain).   In addition, data have been provided in terms of 'total number of immunizations administered rather than in terms of total number of children appropriately immunized - a more difficult but more meaningful number to obtain.
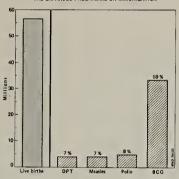
The following assumptions permit a rough estimate to be made of that number: (a) that all reported immunizations were given properly, with potent vaccine, to children who were susceptible to the disease in question; (b) that individual children received a series of three DPT and three poliomyelitis immunizations and one measles or BCG immunization; and (c) that, in continuing programmes, the annual number of births provides an estimate of the size of the target population for these immunizations.

Fig. 1 portrays the application of these assumptions to the available data, and indicates the unsatisfactory level of immunization coverage that was being achieved in these countries during the pre-EPI periods generally covered by the reports.[1]   It should be emphasized that the assumptions used provide data which undoubtedly err in showing higher coverage rates of

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[1] Figs 1-7 are based on the latest complete-year reports available from the countries during the period 1974-1977.

susceptible children than were actually being achieved.   (No estimate has been provided for tetanus vaccination coverage, since the reported data relate to vaccination and revaccination of the entire adult population rather than to the immunization of pregnant women, the group of highest priority for preventing neonatal and puerperal tetanus.)

FIG. 1

COMPARISON OF ESTIMATED ANNUAL LIVE BIRTHS
AND IMMUNIZATION COVERAGE FOR
42 COUNTRIES ACTIVELY PARTICIPATING IN
THE EXPANDED PROGRAMME ON IMMUNIZATION

FIG. 2 – 7
REGIONAL COMPARISONS OF ESTIMATED ANNUAL LIVE BIRTHS
AND IMMUNIZATION COVERAGE BASED ON DATA REPORTED TO WHO

Figs 2-7 use these same assumptions to estimate the immunization coverage being achieved by region - all countries, whether or not collaborating with WHO in expanding their programmes, being included.

A comparison of reported disease incidence rates in the 42 countries identified as expanding their immunization programmes in active collaboration with WHO, and 20 industrialized countries of Europe and North America, is presented in Table 1.

A pattern is seen: the largest differences in reported incidence rates are observed with poliomyelitis (53 times less in the industrialized countries), tetanus (35 times less) and diphtheria (18 times less).   These are underestimates of the actual differences, as only a low proportion of the cases is being reported from the "EPI" countries.   (This is a similar situation to that observed during the early years of the smallpox eradication programme, when it was discovered that less than 10% of the cases were being recorded through the existing reporting systems.)   The rates reflect the effectiveness of current immunization programmes in the industrialized countries.

TABLE 1.   COMPARATIVE INCIDENCE RATES PER 100 000 POPULATION
(Based on data available to WHO as of February 1978)

	Popula-tion (thousands)	Diph-theria	Pertussis	Tetanus	Measles	Polio-myelitis	Tuber-culosis
20 industrialized countries in Europe and North America[1]	856 333	0.09	7.5	0.07	92.57	0.03	21.93
42 developing countries actively participating in EPI[2]	1 289 583	1.61	13.49	2.44	48.35	1.59	34.92

For pertussis (whooping cough) the data are less dramatic, showing a reported incidence for the industrialized countries slightly more than half of that of the "EPI" countries.   In both industrialized and "EPI" countries, the reported pertussis incidence is higher than for polio, diphtheria and tetanus, and this despite the fact that pertussis vaccine is mostly administered with diphtheria and tetanus toxoids as DPT vaccine.   This underlines the point that because pertussis often attacks children early in life, and because the vaccine itself is not as stable or as effective as are diphtheria and tetanus toxoids, this disease is proving more difficult to control in many countries of the world.
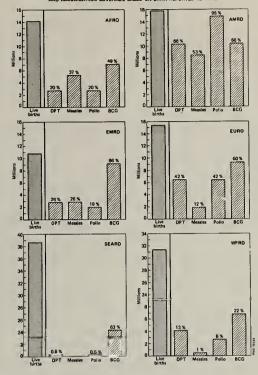
The reported incidence of tuberculosis is high, with underreporting masking the true severity of this problem in the "EPI" countries.   Although these countries report a relatively large number of BCG vaccinations, they are given to older as well as younger children, and a proportion represent revaccinations.   This makes the true coverage of susceptible persons much lower than the 59% shown in Fig. 1.   In addition, tuberculosis is not a disease confined to children, and the impact on reported disease incidence can be expected to take place much later after the time of BCG vaccination than is the case with the other diseases in question.   It should also be emphasized that BCG vaccination is but one element in tuberculosis control programmes.

Measles appears in Table 1 as the disease of greatest reported incidence from both industrialized and "EPI" countries.   The vaccine itself is highly effective, but it is not being extensively utilized in all the industrialized countries, thus accounting for the high incidence.   The underreporting of measles in the "EPI" countries is such that, although they are shown in Table 1 as having an incidence rate lower than that indicated for the industrialized countries, their actual rate is most certainly higher.

3.   COLLABORATING AGENCIES AND ORGANIZATIONS

For its success, EPI depends on the support and collaboration of individuals and groups from the public and private sectors, including voluntary support.   The contributions of a few donors have been summarized in section 4 of this report, which deals with resources which have been made available directly to WHO.   Yet the majority of the contributions are not reflected in that section, and consist of support given direct to country programmes (such as that provided by UNICEF or UNDP, or by donor countries on a bilateral basis) or the support contributed in time, advice and materials by individual concerned citizens, as well as by

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[1] Austria, Belgium, Canada, Czechoslovakia, Denmark, Finland, France, German Democratic Republic, Germany, Federal Republic of, Hungary, Ireland, Italy, Netherlands, Norway, Poland, Sweden, Switzerland, United Kingdom, United States of America, and the USSR.
[2] See footnote, page 4.

concerned professionals and their parent institutions.   Those institutions which have been particularly active at the global level in this last respect include the following:

Royal Tropical Institute
Amsterdam, Netherlands

Center for Disease Control
Atlanta, United States of America

Institute for Serobacteriological
Production and Research
Budapest

London School of Hygiene and Tropical Medicine
London

National Institute for Biological Standards
and Control
London

Gamaleja Institute
Moscow

Medical Research Centre
Nairobi

Institut Pasteur
Paris

National Engineering Laboratory
Stockholm

State Institute for Public Health
Bilthoven, Netherlands

National Institute of Hygiene
Budapest

Technology Consultancy Centre
Kumasi, Ghana

Intermediate Technology Development
Group Ltd
London

Appropriate Health Resources and Technologies
Action Group
London

Mechnikov Research Institute for Vaccine
and Sera
Moscow

International Children's Centre
Paris

National Bacteriological Laboratory
Stockholm

Institute of Immunology
Zagreb, Yugoslavia

In addition to the above institutions, many nongovernmental organizations are collaborating with WHO and other international organizations in initiatives relating to the delivery of primary health care or to the International Year of the Child (1979).   Both initiatives encompass support to immunization activities, and EPI is being promoted by a wide variety of groups through such collaboration.

4.   RESOURCES

Although the support of WHO personnel engaged in EPI activities is being provided from the regular budget, including inputs from the Director-General's and Regional Directors' Development Programmes, programme support in the areas of training and scientific, technological, and operational research and development, and for the major part of the supplies and equipment required for actual programme implementation, must be provided from extra-budgetary sources.   For this reason the Thirtieth World Health Assembly urged "governments and agencies in a position to contribute funds or their equivalent in equipment and supplies . . . to provide maximum longterm support through the Voluntary Fund for Health Promotion . . . or on a bilateral basis . . .".

As summarized below, contributions to date have been encouraging and have supported the necessary global and regional activities in the areas of training, cold-chain development, and vaccine improvement.   However, as more and more countries actively expand their immunization coverage on the basis of well-prepared long-term plans of operation, a much larger level of input will be required to initiate, and - more important - to sustain, this expansion.   For the foreseeable future, the costs of full immunization coverage of the infant population in most of those countries now planning expansion of such coverage is well beyond their limited health budgets.   Contributions on either a multilateral or bilateral basis, and over a five-to ten-year period, will be required by these countries if immunization coverage rates are to be raised to an acceptable level.

4.1 Government contributions to the Special Account for the Expanded Programme on
Immunization of the Voluntary Fund for Health Promotion have been received or pledged to date
as follows:

	1975 US $	1976 US $	1977 US $	1978 US $
Unspecified activities				
Botswana	5 000			
Netherlands	183 889			
Nigeria		16 036		
Saudi Arabia		10 000		
Vaccine				
Finland				36 057
Switzerland			7 843	
United Kingdom			96 590	364 000
Yugoslavia		28 730	33 813	25 857
Specified activities				
Iran – for Pakistan		344 876		
Kuwait – for Democratic Yemen,				
Somalia, and Sudan		150 000	75 000	275 000
Sweden – feasibility studies				
in Ghana	105 890	82 898	37 422	
United Kingdom – cold chain				
equipment evaluation				156 000

4.2 From nongovernmental sources, significant support was received in 1977 from:

Japan Shipbuilding Industry Foundation (Sasakawa Health Fund) . $ 80 000

Professor I. Doğramacı, Ankara 20 000

MEDIMPEX (Hungary) . . . .   7 960 (vaccine)

4.3 In addition to the above contributions specifically recorded under the Special Account
for the Expanded Programme on Immunization, two sources of funding have been responsible for
most of the activities concerned with training and vaccine quality control:

 – DANIDA (Denmark), through contributions to the Special Account for Miscellaneous
   Designated Contributions, has been the main support of the training activities described
   above.   Through 1977, $ 116 184 had been earmarked for these activities and for 1978–
   1979 an amount of $ 391 200 has been placed at the disposition of EPI for the
   preparation and organization of interregional, intercountry and national training
   courses and workshops.

 – UNDP has to date provided an amount of $ 237 200 for an interregional project the
   object of which is to provide the developing countries with vaccines of good potency
   and acceptably low toxicity, and vaccines with increased stability at high ambient
   temperatures.   UNDP is also considering a proposal to extend the above project, and
   regional proposals from the Regional Offices for Africa and South-East Asia for the
   development of programme implementation.

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Nature and Rates of Adverse Reactions Associated with Pertussis Immunization

L. J. Baraff and J. D. Cherry

ABSTRACT

The Pertussis Vaccine Project is designed to accurately determine the rates, nature, and etiology of adverse reactions associated with the administration of DTP vaccine. Fifty thousand DTP immunizations will be prospectively evaluated over a 2 year period. The results of the preliminary studies are presented here.

Children, ages 0–5 years, received either DTP adsorbed or DT adsorbed vaccine and were prospectively evaluated by mail-in questionnaire or home visit for reactions occurring within 48 hours of immunization. Trivalent oral polio vaccine was administered simultaneously to 97% of vaccine recipients.

Reactions were more frequent following DTP vaccine. The ratio of reaction rates associated with DTP and DT immunizations (DTP/DT) for selected local and systemic reactions is reported.

The most striking finding in this preliminary report is the relatively high frequency of persistent crying, convulsion-like episodes, and collapse following DTP immunization.

INTRODUCTION

Prior to 1900, pertussis was a significant cause of infant morbidity and mortality in the United States, but its incidence declined dramatically after pertussis immunization became widespread in the 1950s. Serious adverse effects have been associated with the use of this vaccine since its introduction. However, there are no prospective studies that provide accurate data regarding the rates of either serious or minor adverse reactions associated with the use of vaccines currently licensed in the United States. Several investigators in other countries have suggested that with the declining incidence of pertussis, the rates of serious adverse reactions estimated by retrospective studies make routine pertussis vaccine administration inadvisable.

Recently, great progress has been made in isolating components of *Bordetella pertussis* that may be immunogenic and protective, yet lack reactogenicity. If the adverse reaction rate associated with the current vaccines is sufficiently high, further research efforts should be directed toward the production of a more highly purified vaccine. Therefore this study was undertaken to accurately and prospectively determine the rates of both minor and major adverse reactions associated with the use of current pertussis vaccines.

MATERIALS AND METHODS

Infants and children, aged 6 weeks to 6 years, were enrolled in the study with the informed consent of their parent or guardian. Patients were recruited from the Marion Davies Well-Baby Clinic at the University of California, Los Angeles, the private practices of the clinical faculty of the Department of Pediatrics of UCLA, the Well-Baby Clinics of the Kaiser Hospitals in Los Angeles County, and several Los Angeles County Immunization Clinics. Children who had previously experienced severe adverse reactions following DTP immunization were excluded from the study and only children who would normally have received DTP immunization were included. Immunizations were given according to the schedule and recommendations of the American Academy of Pediatrics. All children received commercially available Diphtheria and Tetanus Toxoids with Pertussis Vaccine Adsorbed (DTP). Ninety-seven percent of children received a simultaneous trivalent oral polio vaccine.

Patients were divided into two groups: those evaluated by mail-in questionnaire, and those evaluated by home visit. Parents of both groups of patients received a questionnaire on which they were asked to record their child's temperature and note any adverse reactions in the 48 hours following immunization. In addition to temperature, reactions specifically to be noted included anorexia; vomiting; fretfulness, local reactions including redness, swelling, and pain; and more serious reactions including persistent screaming, convulsions, and collapse. Parents in the mail-in group were in-

structed to return the questionnaire in a pread-
dressed and stamped envelope to the Pertussis
Vaccine Project. Parents in the home visit group
were instructed that they and their child would
be visited by a Health Associate approximately 48
hours after immunization. During this visit, the
Health Associate reviews the questionnaire with
the parent to evaluate its accuracy as an instru-
ment and the completeness of the parent's report-
ing.

Also included in the data collected were the date
of birth, sex, and ethnic group of the vaccine re-
cipient, the date and time of immunization, the
manufacturer and lot number, the DTP series
number, and the site of immunization. This infor-
mation, together with the date, time, and nature
of adverse reactions, was transferred from the ques-
tionnaire to a data coding form and then key-
punched to IBM cards. Data processing was per-
formed using the computer facilities at the UCLA
Center for the Health Sciences. Statistical analyses
were performed using either the Yates' Corrected
or Pearson Chi Square Tests.

Ninety subjects from the UCLA Well-Baby
Clinic and one private practice were enrolled in a
control group. Infants in this group received a Diph-
theria and Tetanus Toxoids Combined, Pediatric
Vaccine (DH) in place of one of the primary DTP
immunizations. They received an additional DTP
immunization at 9 months of age. These patients
all received an oral polio vaccine in addition to
their DT vaccine. They were then evaluated by
home visit or mail-in questionnaire as were all
patients in the DTP vaccine group.

From February through September, 1978, 2,390
patients were enrolled in the Pertussis Vaccine
Project. Ninety of these patients were entered in
the DT control group. 1,385 were evaluated by
home visit, and 1,005 by mail-in questionnaire. All
patients who experienced either convulsions or
collapse within 48 hours of immunization were
evaluated by the author (Larry J. Baraff, M.D.). All
with convulsions were referred to a pediatric neu-
rologist for a complete history and physical exami-
nation and electroencephalogram.

The sex and ethnic grouping of DTP and DT
vaccine recipients are presented in Table 1. Males
accounted for 52.4 % of the DTP and 55.1% of the
DT vaccine groups. There were significantly more
minority (black, Spanish-surnamed and other) pa-
tients in the DT than the DTP group (χ^2 = 35.94,
p <0.0001). This reflects the enrolling of patients

Table 1. Patient Demography: DTP vs DT

	DTP	DT
Sex		
Male	1,206 (52.4)[a]	49 (55.1)[a]
Female	1,094 (47.6)	40 (44.9)
Ethnic Group		
White	1,400 (66.6)	34 (46.7)
Black	186 (8.8)	10 (11.2)
Spanish-surnamed	399 (19.0)	38 (42.7)
Other	117 (5.6)	0 (0.0)

[a] Percent of total vaccine group

in the DT group from the UCLA Marion Davies
Children's Clinic. Vaccine reactions have not been
analyzed by ethnic group, so it is not possible to
determine whether differences occur between groups
as a result of ethnic imbalance.

The number of vaccine recipients by series num-
ber for DTP and DT immunizations is given in
Table 2. Three patients were inadvertently enrolled
in the DT control group at the time of their fourth
immunization. Approximately 88% of study pa-
tients were less than 1 year of age.

RESULTS

Reactions have been classified as local, less seri-
ous, and more serious. Tables 3–5 present a com-
parison of the rates of these reactions between the
group of patients who received DTP vaccine and
the control DT group. The p values have been
calculated using Yates' Corrected Chi Square Analy-
sis. Parents and Health Associates were aware of
which immunization the child received at the time
of evaluation.

Local Reactions

Table 3 presents the local reactions for both
DTP and DT vaccine recipients. Local redness,
swelling, and pain were all significantly more fre-
quent (p <0.0001) in the DTP recipient group.

Table 2. Series Number: DTP vs DT

Series Number	DTP	DT
1	813 (35.4)[a]	42 (46.7)[a]
2	648 (28.2)	30 (33.3)
3	556 (24.2)	15 (16.7)
4	257 (11.2)	3 (3.3)
5	22 (1.0)	0 (0.0)

[a] Percent of total vaccine group

Table 3. Local Reactions: DTP vs DT

Reaction	DTP	DT	p
Redness	1174 (51.0)[a]	15 (16.7)[a]	<0.0001
Swelling	1294 (56.3)	15 (16.7)	<0.0001
Pain	1141 (49.7)	17 (18.9)	<0.0001
Number evaluated	2,295–2,300	90	

[a] Percent of total vaccine group

All three reactions occurred in approximately 50% of DTP vaccine recipients and only approximately 18% of DT vaccine recipients. Qualitatively, those local reactions experienced by recipients of DTP vaccine were more severe than in recipients of DT vaccine.

Less Serious General Reactions

Table 4 presents the comparative rates of less serious reactions for both DTP and DT vaccine recipients. Fever, drowsiness, fretfulness, and anorexia were significantly more frequent following DTP immunization. Fever (>37.8° C) occurred in 47.7% of DTP and 10.0% of DT vaccine recipients. Table 5 presents the degree of fever present in both DTP and DT vaccine recipients; 6.2% of DTP recipients had a fever ($\geq$39° C), but no DT recipient had a fever of this degree. Drowsiness occurred in 31.1% and 17.8% of DTP and DT vaccine recipients respectively. Fretfulness occurred in 56.3% and 20.0%, vomiting in 7.3% and 4.4%, and anorexia in 26.2% and 11.1% of DTP and DT recipients respectively.

Serious Reactions

The rates of more serious reactions following DTP and DT immunizations are presented in Table 6. Persistent crying was defined as unusual and persistent crying lasting an hour or longer. It occurred in 5.9% of DTP recipients and 2.2% of DT recipients. The duration of the crying is pre-

Table 4. Less Serious Reactions: DTP vs DT

Reaction	DTP	DT	p
Drowsiness	714(31.1)[a]	16(17.8)	0.0102
Fretfulness	1293(56.3)	18(20.0)	<0.0001
Vomiting	167(7.3)	4(4.4)	0.4177
Anorexia	603(26.2)	10(11.1)	0.0019
Temperature >37.8° C	957(47.7)	7(10.0)	<0.0001
Number evaluated	2,007–2298	70–90	

[a] Percent of total vaccine group

Table 5. Temperature: DTP vs DT

Temperature	DTP	DT
>37.8° C	957(47.7)[a]	7(10.0)
$\geq$38° C	602(30.0)	4(5.7)
$\geq$39° C	102(5.1)	0(0.0)
$\geq$40° C	22(1.1)	0(0.0)
Total	2,007	70

[a] Percent of total vaccine group

sented for both groups in Table 7. Crying lasting 4 hours or more occurred only among DTP vaccine recipients. In many instances, persistent crying was not associated with fever or local reaction.

No DT vaccine recipient experienced convulsion or collapse following immunization. Four DTP recipients had convulsionlike activity following immunization. The families of these four patients were interviewed by the author and all were judged to have had convulsionlike episodes in the 48 hours following DTP immunization. In all instances, this activity resembled a generalized major motor seizure of the tonic-clonic variety. All four infants were less than 7 months of age and all reactions occurred within 24 hours of immunization. In three instances, this reaction occurred following the third DTP; the remaining case occurred following the first DTP. No child had a previous history of any neurologic impairment, including febrile seizures. The duration of the tonic-clonic activity in all patients was from 15–30 seconds. One patient had two, one three, and two four of these brief convulsionlike episodes. Two patients were afebrile, and two had fever of approximately 39° C. All returned to their normal baseline state within 48 hours of immunization, and all were judged to have normal neurological examinations when seen by the author within 2 weeks of their reaction.

The four episodes of collapse were manifest by strikingly decreased spontaneous activity, extremely

Table 6. More Serious Reactions: DTP vs DT

Reaction	DTP	DT	p
Persistent crying	136(5.9)[a]	2(2.2)	0.2124
Convulsions	4(0.2)	0(0.0)	1.0000
Collapse	4(0.2)	0(0.0)	1.0000
Number Evaluated	2,294–2,298	90	

[a] Percent of total vaccine group

Table 7. Persistent Crying: DTP vs DT

Duration of Crying	DTP	DT
≥1.0 hr.	136(5.9)[a]	2(2.2)
≥2.0 hrs.	79(3.4)	1(1.1)
≥4.0 hrs.	27(1.2)	0(0.0)
≥6.0 hrs.	15(0.7)	0(0.0)
≥8.0 hrs.	7(0.3)	0(0.0)
≥10.0 hrs.	4(0.2)	0(0.0)
Total	2,294	90

[a] Percent of total vaccine group

Table 9. DTP: Percent Vaccine Reactions by Series Number

Reaction	n[a]	Series Number 1	2	3	4	5	p
Drowsiness	2,290	36.1	30.2	28.0	24.5	27.3	0.0015
Fretfulness	2,290	56.3	55.9	57.0	57.6	40.9	0.6550
Vomiting	2,290	8.0	8.4	5.2	5.5	13.6	0.0976
Anorexia	2,290	29.6	22.6	22.4	33.1	31.8	0.0005
Crying	2,286	8.4	6.2	3.2	3.9	0.0	0.0006

[a] Number evaluated

poor fluid intake, marked lethargy, and pallor. All patients had a fever greater than 39° C. The duration of these reactions was 3, 15, 17, and 36 hours. All patients returned to their normal level of activity thereafter and when evaluated by a board certified pediatrician were found to be normal. Only one of these patients was seen by a physician during this reaction. The physician's impression was that the child had an encephalitislike syndrome. Two collapselike episodes occurred after the third, and one each after the first and second DTP.

DTP Vaccine Reactions by Series Number

A statistically significant difference for several types of reactions following DTP immunization was noted by immunization series number. The percent of reactions by series number is presented in Tables 8 and 9. The number of patients included for calculation of percentages for each series number is specified. The number of patients who received their fifth DTP immunization is too small for valid statements regarding reaction rates.

In general, local reactions and temperatures were more frequent with increasing series number (Table 8). There was a significant difference in the percentage of patients with all these reactions by series number. However, the difference noted for pain most probably reflects the older child's ability to

vocalize this complaint. In contrast, several generalized reactions were less frequent with increasing series number. Drowsiness, vomiting, and persistent crying were more frequent following the initial immunizations.

Home Visit vs. Mail-In Questionnaires

Reaction rates have been compared by the means of data collection, i.e., home visit versus mail-in questionnaire. There was a significant difference in the rates of reactions for only three variables: temperature, local swelling, and drowsiness. Fever was more common in the mail-in questionnaire group (52.0%) than in the home visit group (41.5%). This probably reflects the fact that some parents in the home visit group had not taken the child's temperature prior to the home visit. Local swelling was more common in the home visit group, as the Health Associates were specifically instructed to feel induration at the site of immunization which might not have been noticed by the parent. Drowsiness occurred in 36.2% of the home visit patients and in 29.3% of the mail-in patients. This difference is unexplained.

DISCUSSION

The results presented here are preliminary and have been gathered during the time that the Pertussis Vaccine Project was recruiting participating physicians and training staff members. However, they are presented because no other prospective data regarding reaction rates associated with the use of U.S. licensed DTP vaccines are available.

Complications following administration of pertussis vaccine were reported almost immediately after Sauer (1) and Kendrick and Eldering (2) produced an effective vaccine. In 1933, Madsen (3) reported two instances of sudden death within hours of pertussis vaccination. Subsequently, there have been many reports of both serious and minor

Table 8. DTP: Percent Vaccine Reactions by Series Number

Reaction	n[a]	Series Number 1	2	3	4	5	p
T° >37.8° C	2,000	27.2	34.6	37.0	48.1	38.9	<0.0001
Redness	2,292	47.2	47.8	57.0	56.4	63.6	0.0006
Swelling	2,292	54.8	52.6	60.3	59.9	72.7	0.0196
Pain	2,287	47.1	44.8	47.4	72.0	86.4	<0.0001

[a] Number evaluated

reactions following pertussis immunization (4–20). At least 250 serious reactions have been reported to date. These include convulsions, hypsarrythemia, shock, coma, persistent screaming, and delirium. The fatality rate associated with such complications has been reported to be from 2–15% (4,5). Serious neurologic sequelae including persistent convulsions, hemiplegia, and psychomotor retardation may occur in as many as 50% of these patients (6), possibly reflecting the bias of this method of case-finding. Other reactions associated with pertussis inoculation include erythema and induration at the site of injection; hyperpyrexia; vomiting and diarrhea; edema of the hands, face, and feet; and various exanthums.

In most instances, serious reactions occur within 12 hours of injection. The relationship of those reactions occurring more than 48 hours after injection of the vaccine is uncertain and is more likely to represent coincidence than causal association. Reactions may occur with any injection in the series of primary immunizations, or with booster injections; however, the number of reported reactions decreases with increasing order of injection (4,5).

Though the clustering of serious reactions within hours of immunization suggests an etiologic relationship, some doubt the relationship of vaccination to these reactions. Griffith (21) estimates that in the United Kingdom, the incidence of first convulsions in infants between 6 and 24 months of age, is 3 per 100,000 children per day. This is based on Ounstead's (22) data which demonstrate that between 5–7% of children have at least one convulsion before they are 7 years of age. In the Pertussis Vaccine Trials conducted by the Medical Research Council from 1951–1954, the incidence of post-vaccination convulsion was 1.5/100,000 (7). They concluded that pertussis vaccine was responsible for only a minority of postvaccination convulsions. Helstrom (8), after performing serial pre- and post-vaccine EEGs on 84 children who received pertussis vaccine, found none with EEG changes attributable to vaccination. On the other hand, Strom (4), after conducting a nationwide survey of neurologic complications following pertussis vaccination in Sweden from 1955–1958, reported 167 serious reactions among 516,276 vaccine recipients. The incidence of these reactions was thus 32/100,000 vaccine recipients. Convulsions accounted for 80 of these reactions. Hannik (9) reported 50 major reactions following DTP polio vaccination in the Nether-

lands in a 7-year period during which 1,260,000 children were immunized. Thirteen of these reactions were convulsions. Thus the incidence of serious reactions and convulsions during this period could be crudely estimated to be 3.9/100,000 and 1.0/100,000 respectively. These figures are much lower than those reported by Strom, but only spontaneously reported cases were tabulated.

The most striking findings in this preliminary report are the relatively high frequency of persistent crying, convulsionlike episodes, and collapse following DTP immunization. The significance of persistent crying is unknown. Possible etiologies for this reaction are severe pain due to local irritation produced by the vaccine or response to central nervous system irritation. The fact that many children who manifest persistent crying have no evidence of local reaction suggests that the latter may be responsible for some of these reactions.

To date, all of the convulsionlike episodes associated with vaccine administration have been brief (<30 seconds) and have not been followed by evidence of neurologic impairment. However, the fact that such reactions have occurred in the absence of fever is evidence for potential neurotoxicity of this vaccine. A larger number of observations will be required to substantiate this initial impression. Four children developed collapse following DTP immunization; these reactions were dramatic and are of unexplained etiology.

The mechanisms of untoward reactions to pertussis vaccination in man are unknown. Immediate reactions such as fever, convulsion, and shock may be due to direct toxic effects of the vaccine, either from components of the *Bordetella pertussis,* i.e., endotoxin, or impurities introduced during vaccine manufacture, i.e., media. The production of the current vaccine is primarily in artificial media free of animal products. *Bordetella pertussis* is known to contain many biologically active substances including eight agglutinogens, a hemagglutinating substance, endotoxin, heat labile toxin, mouse protective antigen, lymphocyte promoting factor, and heat stable antigen activity. Several of these properties may be associated with one substance. One of these biologically active substances present in the vaccine is most likely responsible for adverse reactions. For example, convulsions may be due to hypoglycemia induced by insulin stimulating activity present in the vaccine.

In any discussion of DTP vaccine reactions, it is important to note that not all manufacturers pro-

duce this vaccine by similar processess. There is in fact, considerable variation in biological activity from lot to lot produced by any given manufacturer. Therefore, adverse reactions may reflect the lack of standardization in the production of this vaccine or excess toxicity of vaccine produced by one manufacturing process, rather than the inherent toxicity of all DTP vaccines. We are in the process of evaluating differences in reaction rates by manufacturer and lot number.

ACKNOWLEDGMENTS

REFERENCES

1. Sauer, L. Whooping cough: a study in immunization. J.A.M.A. 100:239; 1933.
2. Kendrick, P.; Eldering, G. A study in active immunization against pertussis. Am. J. Hyg. 29:133; 1937.
3. Madsen, T. Vaccination against whooping cough. J.A.M.A. 101:187–188; 1933.
4. Strom, J. Further experience of reactions, especially of a cerebral nature, in conjunction with triple vaccination: a study based on vaccinations in Sweden 1959–1965. Med. J. 4:320–323; 1967.
5. Berg, J. M. Neurological complications of pertussis immunization. Br. Med. J. 2:24–27; 1958.
6. Aicardi, J. Accidents neurologiques consecutifs a la vaccination contre le coqueluche. Arch. Franc. Pediatr. 32:309–315; 1975.
7. Vaccination against whooping cough: relation between protection in children and results of laboratory tests. A report to the Whooping Cough Immunization Committee of the Medical Research Council and to the Medical Officers of Health for Cardiff, Leeds, Leyton, Manchester, Middlesex, Oxford, Poole, Tootenham, Walthamstow, and Wembley. Br. Med. J. 2:454–462; 1956.
8. Panel discussion on immunizations in infants and small children. Proceedings of the Swedish Pediatric Society. Acta Paediatr. 51:232–237; 1962.
9. Hannik, C. A. Major reactions after DTP-polio vaccination in the Netherlands. International Symposium on Pertussis. Basel: Karger; 1970; 157–161.
10. Byers, R. K.; Moll, M. D. Encephalopathies following prophylactic pertussis vaccine. Pediatrics 1:437–457; 1948.
11. Kulenkampff, M.; Schwartzman, J. S.; Wilson, J. Neurological complications of pertussis inoculation. Arch. Dis. Child. 49:46–49; 1974.
12. Toomey, J. A. Reactions to pertussis vaccine. J.A.M.A. 139:448–450; 1949.
13. Kong, E. Zur Pertussisimpfung und ihren Gegendindikationen. Helv. Paediatr. Acta 8:90–98; 1953.
14. Globus, J. H.; Kohn, J. L. Encephalopathy following pertussis vaccine prophylaxis. J.A.M.A. 141:507–509; 1949.
15. Melin, K. A. Pertussis immunization in children with convulsive disorders. J. Pediatr. 43:652–654; 1953.
16. Miller, H. G.; Stanton, J. B. Neurological sequelae of prophylactic inoculation. Q. J. Med. 23:1–27; 1954.
17. Beaudoing, A.; Gilbert, Y.; Butin, L. P.; Jaillard, M. Complication nerveuses ou decours d'une vaccination associee. Pediatrie 19:1004–1009; 1964.
18. Thursby-Pelham, D. C.; Files, C. Neurological complications of pertussis immunization. Br. Med. J. 2:246; 1958.
19. Sutherland, J. Encephalopathy following diphtheria pertussis inoculation. Arch. Dis. Child. 28:149–150; 1953.
20. Melchior, J. C. Infantile spasms and immunization in the first year of life. Neuropaediatrie 3:3–10; 1971.
21. Griffith, A. H. Pertussis vaccine and convulsive disorders of childhood. Proc. R. Soc. Med. 67:16–18, 1974.
22. Ounstead, C.; Some aspects of seizure disorders. Gairdner, D.; Hull, D., eds. Recent advances in paediatrics, 4th edition. London: Churchill; 1971; 363.

Changes in Plasma Insulin Concentration and Temperature of Infants After Pertussis Vaccination

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ABSTRACT

Infants injected with DTP-Polio vaccine with a pertussis component of 16 International Opacity Units per dose showed slight but significant elevation in concentration of plasma insulin and temperature. These two phenomena are not interrelated.

It is suggested that infants who show serious reactions following pertussis vaccination suffer from a failure to maintain glucose homeostasis.

INTRODUCTION

There is considerable concern about the reactions that sometimes occur in children following the injection of pertussis vaccine. Reactions have been observed and reported since 1933 (1) and range from the slightest minor reaction to status convulsivus resulting in permanent cerebral damage.

Until now, there has been no explanation for the severe or major reactions or identification of responsible factors in pertussis vaccine. Nor is there a satisfactory laboratory method of testing vaccines for reactivity before they are used in children. In the Netherlands all pertussis vaccines pass the mouse weight gain test for vaccine toxicity according to the WHO recommendations (2). Although there is some relation between the results of this test and the incidence and degree of minor reactions in children (3,4), there is no evidence of a relationship with the incidence of major reactions.

Laboratory investigations have shown that injection of pertussis vaccine into experimental animals not only results in protection against an intracerebral challenge with *B. pertussis* and sensitization against histamine but also affects carbohydrate and fat metabolism.

Pertussis vaccine induces hypoglycemia in mice (5) and attenuates the marked hyperglycemia elicited by adrenaline (6) in mice. The level of free fatty acids in rats is not changed by pertussis vaccination; but in vitro fat pads from vaccinated rats show marked lipolysis, which drew the attention of Tabachnick and Gulbenkian (7, 8) to insulin. They demonstrated that an injection of pertussis vaccine into rats results in a markedly elevated level of circulatory immunoreactive insulin and a reduction in the blood sugar level, although not to the extent that would have been expected from the degree of increase in insulin concentration.

The observation of mixed reactions—combinations of screaming, shock, and convulsions—in a child strongly points to one toxic factor in pertussis vaccine. Each of the various reactions, actually symptoms, might be caused by hypoglycemia. There is some evidence that there may be a failure in some young children to maintain glucose homeostasis following pertussis vaccination. A low blood sugar level (9) and an extremely low CSF-glucose concentration (10) have been reported in children who developed convulsions 3 and 36 hours after receiving pertussis vaccine.

In view of these findings we studied a small number of babies to determine the effect of injections of pertussis vaccine on plasma insulin and blood sugar concentration and also recorded the temperatures of some of the babies.

MATERIALS AND METHODS

Three different polyvalent vaccines, designated A, B, and C, were used, each with identical content of diphtheria, tetanus, and inactivated polio virus components. All contained 1.5 mg aluminum phosphate per ml dose. Vaccines A and B also contained pertussis component, with concentrations of organisms per dose equivalent to 16 International Opacity Units (OU) for vaccine A and 10 OU for vaccine B. Vaccines A and B each had an immunizing potency for the pertussis component of not less than 4 IU per dose, which is the minimal value

required by the WHO (2). Vaccine C contained no pertussis component.

Fifty-four healthy babies who were being fed five times a day at regular intervals of 4 hours were included in the study. Informed consent was obtained from their parents. The babies were randomly assigned to four groups: Group I received vaccine A (DTP-Polio, 16 OU); Group II received vaccine B (DTP-Polio, 10 OU); Group III received vaccine C (DT-Polio, no pertussis component); Group IV received no vaccination.

The mean age of the babies was 14.5 weeks and the variation between the means of the four groups was 0.6 week. The vaccine was injected intramuscularly 2 hours after the first feeding, and blood samples were taken 8 hours after vaccination, i.e., 2 hours after the third feeding. Blood samples were taken at the same time from the nonvaccinated control group. Samples were tested for plasma insulin concentration by radioimmunoassay, using the BRC standard for human insulin, No. 66-304, as reference.[1] The blood sugar concentration was measured by the glucose oxidase method.[2]

The study was preceded by a number of tests which showed that babies 3 to 4 months old receiving five feedings a day at regular intervals of 4 hours do not have a diurnal rhythm of plasma insulin concentration.

Rectal temperatures of 43 of the 54 children were also recorded just before the blood samples were taken. All measurements were performed with one thermometer.

RESULTS

There were no changes in the blood sugar level in any of the children 8 hours after vaccination with any of the three vaccines (Table 1). There

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[1] Determination under supervision of Dr. W. H. L. Hackeng of the Laboratory for Endocrinological Chemistry, Bergweg Hospital, Rotterdam; Head, Dr. W. Schopman.

[2] Determination under supervision of Miss A. Schaller of the Laboratory for Clinical Chemistry, Bergweg Hospital, Rotterdam; Head, Dr. G. Korevaar.

Table 1. Blood-Sugar Concentration in mg/100 ml, 8 Hours After Vaccination

Vaccine	Number	Mean Level
DTP-Polio, 16 OU	14	76.4
DTP-Polio, 10 OU	16	81.0
DT-Polio	14	72.2
Control	9	76.6

Table 2. Plasma Insulin Level in u/ml 8 Hours After Vaccination

Vaccine	Number	Mean Level
DTP-Polio, 16 OU	14	13.5
DTP-Polio, 10 OU	16	8.9
DT-Polio	14	6.5
Control	10	7.1

were, however, significant changes in the plasma insulin concentration, as shown in Table 2 (Student's test, one-sided level of significance 5%).

A slight but significant elevation of the plasma insulin level was found in children who received vaccine A (16 OU per dose) as compared with those who received vaccine C (no pertussis component) as well as those in the unvaccinated control group (Student's test, one-sided level of significance 5%).

The results with the group given vaccine C showed no significant difference from those with the unvaccinated group.

The group receiving vaccine B (10 OU per dose) showed some insulin elevation. Although the value was intermediate between those obtained with vaccine A and the control group, the differences were not significant.

Both vaccines A and B, containing pertussis component, induced significantly elevated mean temperatures, whereas vaccine C, containing no pertussis component, induced no change, the results being the same as with the control group (Table 3). Moreover, the group receiving vaccine A showed a significantly higher elevation of temperature than the group receiving vaccine B.

No relationship could be established between the elevation of plasma insulin and temperature in individual children, and no major reactions were observed in any of the children.

DISCUSSION

Injection of pertussis vaccine in rats results in a much higher elevation of the plasma insulin level than an injection of DTP-Polio vaccine, 16 OU,

Table 3. Temperature in °C 8 Hours After Vaccination

Vaccine	Number	Mean Temperature
DTP-Polio, 16 OU	11	38.3
DTP-Polio, 10 OU	15	38.1
DT-Polio	9	37.4
Control	8	37.4

in infants. This might be because the dose per kg body weight is much higher for rats than for infants. A further difference is the time of blood sampling. In most laboratory investigations on rats sampling is started 24 hours after vaccination and continues for some weeks. In this study we chose an interval of 8 hours after injection because most major reactions occur within 12 hours. We thought it more important to look for a possible early imbalance of carbohydrate metabolism rather than wait for 24 hours, during which the imbalance might disappear.

The babies succeeded in maintaining their glucose homeostasis. The attenuation of the hyperglycemia elicited by adrenaline probably would have been a better measurement of the effect of pertussis vaccine on carbohydrate metabolism, but we did not consider such a study to be justified.

The hypothesis that major reactions might be associated with hypoglycemia is not supported by these results, but in our opinion it is still worth further study on larger groups of infants. Such a study might be carried out in countries still using vaccines with a higher concentration of pertussis component, such as 20 OU per dose, for it is with such vaccines that conclusive results are more likely to be obtained.

So far, the elevation of the plasma insulin level has been demonstrated to be one untoward effect of pertussis injection both in infants and in rats. It is a parameter that can be measured accurately.

Another symptom of toxicity in man is temperature elevation. Usually, this measurement is performed about 24 hours after vaccination, when it is a good indicator of minor reactions. Quite healthy babies also show a temperature of about 38°C about 8 hours after injection.

The rise in plasma insulin level and temperature are not related, which suggests a different etiology for each phenomenon.

Most studies of pertussis vaccine have been done in the laboratory. However, the clinical approach to studying effects of pertussis vaccine other than protection and untoward reactions deserves more attention, particularly in view of the possible development of purified vaccine in the near future.

REFERENCES

1. Madsen, T. Vaccination against whooping cough. J.A.M.A. 101:187–188; 1933.
2. Requirements for pertussis vaccine. W.H.O. Tech. Rep. Ser. 274:25–40; 1964.
3. Cohen, H.; van Ramshorst, J. D.; Drion, E. F. Relation between toxicity tests in mice and reactions in children using four lots of quadruple vaccine (DPT-polio). Symp. Series Immunobiol. Standard. 10:53–62; 1969.
4. Perkins, F. T.; Sheffield, F.; Miller, Chr. L.; Skegg, J. L. The comparison of toxicity of pertussis vaccines in children and mice. Symp. Series Immunobiol. Standard. 13:141–149; 1970.
5. Parfentjev, I. A.; Schleyer, W. L. Influence of histamine on the blood-sugar level of normal and sensitized mice. Arch. Biochem. 20:341–346; 1949.
6. Szentivanyi, A.; Fishel, C. W.; Talmage, D. W. Adrenaline mediation of histamine and serotonin hyperglycemia in normal mice and the absence of adrenaline-induced hyperglycemia in pertussis sensitized mice. J. Infect. Dis. 113:86–98; 1963.
7. Tabachnick, I. I. A.; Gulbenkian, A. Mechanism of diazoxide hyperglycemia in animals. Ann. N. Y. Acad. Sci. 150:204–218; 1968.
8. Tabachnick, I. I. A.; Gulbenkian, A. Adrenergic changes due to pertussis: insulin, glucose and free fatty acids. Eur. J. Pharmacol. 7(2):186–195; 1969.
9. Globus, J. H.; Kohn, J. L. Encephalopathy following pertussis vaccine prophylaxis. J.A.M.A. 141:507–509; 1949.
10. Anderson, I. M.; Morris, D. Encephalopathy after combined diphtheria-pertussis inoculation. Lancet 258:537–539; 1950.

Reactions to DTP Vaccine, by Lot and Manufacturer: Results of a Survey in Montana

R. S. Hopkins

ABSTRACT

Reports to the Montana State Health Department in November 1977 of apparent excessive reactions after administration of one lot of a manufacturer's DTP-adsorbed vaccine prompted a telephone survey of DTP vaccine recipients. In the 10 months from November 1977 to August 1978, a single interviewer contacted parents of 227 children who had been vaccinated at four different clinics with one of six lots of DTP vaccine produced by two manufacturers. None of the children had received other injections at the same visit. The mean age of the children was 18 months.

A reaction was defined as fever lasting more than 36 hours, or temperature over 100.8°F (38.2°C) when measured, or inflammation or ecchymosis at the injection site lasting 24 hours or more.

Reaction rates for individual lots of vaccine ranged from 4.2% to 32.5%. Studied lots of Lederle and Wyeth vaccine all met FDA criteria for safety and potency. Mouse pertussis protection bioassay values were positively correlated with reaction rates, but mouse toxicity values were not.

INTRODUCTION

In early November 1977, a county public health nurse telephoned the Preventive Health Services Bureau, Montana Department of Health and Environmental Sciences, to report that three of eight children given DTP vaccine at her most recent immunization clinic had developed unusually severe reactions. These were characterized by high fever; marked ecchymosis; and swelling, warmth, and pain at the injection site. A few days later, a nurse at a busy private pediatric clinic reported that the number of telephone calls from parents concerned about DTP vaccine reactions had gone from one or two a week to one or two a day when her clinic switched from Lilly vaccine to Lederle vaccine. The number of calls fell to previous levels when, after a 4 week trial of Lederle vaccine, the clinic switched to Wyeth DTP vaccine.

At the time the increase in reactions was noted, both clinics had been using Lederle vaccine lot #510–234. A check with Lederle headquarters showed that 14,377 10-dose vials of this lot had been manufactured since July 1977 and that this was the principal lot being shipped from the West Coast at that time.

Many local health departments in Montana receive vaccine from the state health departments, which had been supplying Lederle lot #510–234. Many, however, buy their own vaccine. Conse-

quently, it was possible to design a retrospective telephone questionnaire study to compare reaction rates for several different manufacturers' vaccines. We attempted to answer two questions: Was the apparent increase in reactions after Lederle vaccine real? If so, was it a property only of Lederle lot #510–234 or of all lots of Lederle vaccine?

METHODS

In November 1977, five public clinics supplied lists of children who had been vaccinated 1–6 weeks previously with DTP but with no other injectable vaccine. One clinic (K) had just switched from Wyeth lot #62112 to Lederle lot #510–234 (the suspected lot). Clinic K was studied again in January 1978, when it was using Lederle lot #510–269, and in August 1978, when it was using Wyeth vaccine lot #63701. Three other clinics that had used two other lots of Lederle and two lots of Wyeth vaccine were also studied (see Table 2).

The following information was obtained from each child's record: age, sex, previous DTP immunizations, other vaccines given at the same visit, and parents' telephone number.

All of the telephoning was done by one interviewer. The average call took less than 60 seconds. The interviewer asked about reactions or illness in the 48 hours after vaccine administration, first as an open-ended question, then specifically as to

Table 1. Reaction Grading for DTP Vaccine Reaction Survey

I. No reaction reported.
II. Mild reaction: fever lasting up to 36 hours and a temperature of <101°F (38.3°C) when measured. Minimal physical findings at injection site.
III. Moderate reaction: fever lasting more than 36 hours, or a temperature ≥101°F (38.3°C) when measured. Physical findings at injection site: erythema or swelling or warmth lasting ≥24 hours. Noticeable limp lasting ≥48 hours.
IV. Marked reaction, two or more of the following: erythema, induration, local heat, or ecchymosis at the injection site, with or without fever, lasting ≥24 hours.

fever and its duration, local reaction at the site of injection and its duration, and degree of disability (for example, limp or inability to walk in a toddler).

Overall reactions were graded as shown in Table 1, using criteria related to both fever and local reactions. A reaction grading system using local reactions alone yields very similar results on analysis. This system is somewhat arbitrary but has the advantage in a telephone interview of emphasizing duration of physical findings rather than details of their appearance.

For most analytic purposes reaction rate groups I and II were combined, as were groups III and IV, so that a child was said to have a reaction when he or she had fever lasting more than 36 hours, or a temperature of 101° F (38.3° C) or over when measured, or local inflammation or ecchymosis at the injection site lasting 24 hours or more, or a noticeable limp lasting 24 hours or more.

RESULTS

From November 1977 to August 1978, a single interviewer contacted parents of 227 children who had been vaccinated at four different clinics with one of six lots of DTP vaccine produced by two manufacturers. All parents contacted by telephone were successfully interviewed (Table 2). Ninety-two percent of parents with telephone service were contacted, and 70.3% of all children vaccinated were followed up. The median age of the subjects ranged from 6 months at two clinics to 18 months at one. All but five children in the study received trivalent oral polio and DTP vaccines at the same visit; none received MMR or other injectable vaccines.

Reaction rates (grade III and IV) ranged from 1 of 24 (4.2%) for clinic K with Wyeth lot #62112 to 13 of 40 (35.5%) for clinic K with Wyeth lot #63701. The reaction rate for all groups combined was 45 of 227 (19.8%) (Table 3).

The differences in reaction rates between the various vaccines is statistically significant, and the distribution of reaction rates among the six vaccine lots (combining Wyeth lots #61929 and #62112) is significantly different from a random distribution ($\chi^2 = 10.03$, 4 d.f., p <0.05).

When only children age 13 months or younger are considered (Table 4), similar results are obtained: reaction rates range from 0 of 16 (Wyeth lot #62112) to 10 of 28 (35.7%) Lederle lot #510–234. Wyeth lot #63701 was associated with a reaction rate of 10 of 18 (55.6%) for children ages 13 months or older who were receiving their fourth or fifth dose of DTP vaccine, but only 3 of 22 (13.6%) for children 12 months or younger receiving their first, second, or third DTP shot ($\chi^2 = 6.13$, p <0.02).

Data were obtained from the Bureau of Biologics, Food and Drug Administration (FDA), on safety and potency testing of the specific lots of vaccine used. All lots met FDA requirements for sterility, diphtheria toxoid potency, tetanus toxoid potency, thimerosal content, adjuvant content, and formal-

Table 2. Study Population for DTP-Vaccine-Reaction Survey, Montana, November 1977–August 1978

Clinic, Manufacturer, Lot No.	Number Vaccinated	Number with Telephone	Number Contacted	Vaccinees Contacted %	Vaccinees with Telephones Contacted %	Median Age (months)
B—Lederle 510–234	29	26	24	82.7	92.3	12
G—Lederle 498–608	39	35	33	84.6	94.3	18
H—Wyeth 62112 or 61929	42	35	29	69.0	82.8	12
K—Wyeth 62112	44	26	24	54.5	92.3	6
K—Lederle 510–234	30	28	26	86.7	92.9	7
K—Lederle 510–269	61	52	51	83.6	98.1	6
K—Wyeth 63701	53	45	40	75.5	88.9	9
Total	298	247	227	76.3	92.0	

Table 3. Reaction Rates

Clinic, Manufacturer, Lot No.	Reaction Grade		
	I & II	III & IV	Total
B—Lederle 510–234	17	7 (29.5%)	24
G—Lederle 498–608	28	5 (15.2%)	33
H—Wyeth 62112 and 61929	27	2 (6.9%)	29
K—Wyeth 61112	23	1 (4.2%)	24
K—Lederle 510–234	20	6 (23.1%)	26
K—Lederle 510–269	40	11 (21.6%)	51
K—Wyeth 63701	27	13 (32.5%)	40
Total	182	45	227 (19.8%)

Table 4. Reaction Rates in Children Age <12 Months

Clinic, Manufacturer, Lot No.	Reaction Grade		
	I & II	III & IV	Total
B—Lederle 510–234	7	5 (41.6%)	12
G—Lederle 498–608	14	2 (12.5%)	16
H—Wyeth 62112 and 61929	16	1 (5.9%)	17
K—Wyeth 62112	16	0 0	16
K—Lederle 510–234	11	5 (31.25%)	16
K—Lederle 510–269	32	6 (15.8%)	38
K—Wyeth 63701	19	3 (13.6%)	22
Total	115	22	137 (16.1%)

dehyde content. As shown in Table 5, the pertussis potencies, measured as pertussis units per total human dose, varied considerably from lot to lot and seemed to bear a rough relationship to the rate of reactions seen in this study (r = 0.45, p > 0.1). There is a low negative correlation between pertussis toxicity and reaction rate as measured by average weight gain for mice at 7 days.

DISCUSSION

Among the five lots of DTP vaccine that we studied, the one that prompted the study, Lederle #510–234, did not produce the highest reaction rate, but it had a higher reaction rate than the other two lots of Lederle vaccine studied. The average reaction rate for Lederle vaccine was only slightly higher than that for Wyeth vaccine.

Published data on vaccine reactions, by manufacturer and by lot, are sparse. The difficulties of retrospectively collecting such data have been well described by Griffith (1). The Final Report to the Whooping-Cough Immunisation Committee of the British Medical Research Council in 1959 (2) describes an increased rate of local and febrile reactions for a vaccine prepared from "Pillemer's Antigen" when compared with a vaccine of similar mouse-protection potency prepared from whole cells; 57–73% of children had local erythema at least 1 inch in diameter 48–72 hours after inoculation. Reactions were not otherwise classified by vaccine type.

In a questionnaire survey of parents of 621 DTP vaccine recipients, Barkin and Pichichero (3) recently found that 75.4% of the recipients of an unidentified DTP vaccine had local reactions (either redness, swelling, or tenderness) and over 50% had a temperature of 100° F (37.8° C) or higher.

Weihl, Riley, and Lapin (4) in 1962 compared local and systemic reactions to an unidentified DTP vaccine that contained whole cell (WC) pertussis vaccine and to Lilly vaccine made from extracted pertussis antigen (EPA); 346 children received 1,020 doses of vaccine. Febrile reactions (>0.5° F above normal) were noted for 51% of WC vaccine doses and for 12% of EPA vaccine doses. Only 13% of EPA vaccine recipients had induration, erythema, or edema, as compared to 45% of WC vaccine recipients.

There has been no standardized method of evaluating mild local and febrile reactions to DTP vaccine. The range and frequency of such reactions

Table 5. Pertussis Vaccine Potency and Toxicity, Montana, November 1977–August 1978

Vaccine Mfr. and Lot No.	Children with Grade III or IV Reaction (%)	Potency (Units/THD)		Toxicity in Mice	
		Mfr.	Bureau of Biologics	Avg. wt. gain by day 7 (g)	Died (%)
Lederle 510–234	26.0	33.6	22.9	5.3	0
Lederle 510–269	21.6	—	21.8	6.1	3
Lederle 498–608	15.2	16.3	28.0	7.5	0
Wyeth 62112	4.2	8.6	8.9	8.4	0
Wyeth 63701	32.5	—	18.0	8.1	0
Wyeth 61929 and 62112	6.9	8.6/13.4	8.9/17.5	8.4/7.6	0

for vaccines that meet FDA criteria for mouse toxicity and mouse protection tests have proved acceptable in clinical practice and have not appeared to interfere with the success of immunization programs.

It seems desirable, however, to choose for general use those vaccines that have the lowest local and febrile reaction rates and produce an acceptable level of vaccine efficacy in humans. The British Medical Research Council reported that protection of mice against intracerebral inoculation of pertussis organisms correlated with vaccine efficacy in humans. Still, a fairly wide range of values for mouse protection units/total human dose (8/36) is considered acceptable by the Bureau of Biologics. It is not clear whether values at the upper end of that range really represent significantly greater human vaccine efficacy than values at the lower end.

Data from this study seem to suggest that vaccines with higher mouse protection potencies are associated with higher rates of local reaction. More work is needed to establish whether the upper limit of allowable mouse protection units can safely be lowered to minimize reactions to vaccines. This is important both for prevention of needless morbidity and for the continuing public acceptance of immunization programs.

The telephone survey method described here is rapid and inexpensive and could allow local health departments to determine quickly whether a cluster of local systemic reactions is a chance occurrence, a result of careless vaccine administration at one clinic, or a result of a "hot" lot of one manufacturer's vaccine.

REFERENCES

1. Griffith, A. H. Reactions after pertussis vaccine: a manufacturer's experience and difficulties since 1964. Br. Med. J. 1:809–815; 1970.
2. Whooping Cough Immunisation Committee of the Medical Research Council. Vaccination against whooping-cough. Br. Med. J. 1:994–1000, 1959.
3. Barkin, R. M.; Pichichero, M. E. Diphtheria-pertussis-tetanus: reactogenicity of commercial products. Pediatr. Res. 12:422; 1978.
4. Weihl, C.; Riley, H. D.; Lapin, J. H. Extracted pertussis antigen, a clinical appraisal. Am. J. Dis. Child. 106:124–129; 1963.

DISCUSSION OF PART 5

DR. DUDGEON: As Sir Charles has indicated in his position paper, I am concerned with the assessment of severe reactions to triple vaccine. He referred to a report from my hospital of a number of severely brain-damaged children. This was a retrospective study of children admitted for assessment of neurological defects. After they had been admitted, it was found that they had been immunized with DTP vaccine at some time in the past. They were not admitted as vaccine-damaged children.

Now, as to the question of incidence of vaccine reactions: Dr. Mortimer indicated that the incidences of severe reactions varied from one figure to another. In England, they vary from 1 in 3,000 to 1 in 300,000. We are now trying to do a prospective case control study in which the immunization records of children admitted to hospitals with severe neurological disease are compared with matched children who have no neurological disease.

Figure 1 is a map of the United Kingdom showing the areas from which pediatricians, infectious disease physicians, and neurological surgeons are participating: 580 in all. The study is being run by Professor David Miller at the Central Middlesex Hospital to report neurological illnesses listed in Table 1.

The complications are the following: acute and subacute encephalopathy, convulsions lasting more than 30 minutes, West's syndrome or infantile spasms, Reye's syndrome.

Figure 2 shows what is happening. The physicians notify a patient, aged 2 months to 3 years, who has been admitted with a neurological illness. If a case is accepted (rejection is usually due to the age factor) a questionnaire is sent to the general practitioner and to the specialists in community medicine. At this stage, two controls matched by age and sex are selected. Any child who has recovered completely in 15 days and is discharged, is followed up at intervals through the general practitioner or the consultant. Any child with residual signs 15 days or later is further visited either in the hospital by the team or at home after discharge, and a control child matched by age and sex as far as possible is accepted at that point (Fig. 3) .

In brief summary, after one year of the study, there have been 387 children included in this study, and of these, 255 have been accepted on the grounds of age and neurological illness. These 255 children have been compared with the controls who had no neurological illness.

Table 2 shows the immunization history of the index cases compared with the nonimmunized matched controls. This includes only children receiving DTP vaccine and OPV in the 7 days prior to admission. One additional point is important. In an analysis of 572 of these children, including both cases and matched controls, fairly wide discrepancies were found in the immunization histories which affected 46 percent of the children. The discrep-

Figure 1. Map of the United Kingdom showing the areas from which pediatricians, infectious disease physicians, and neurological surgeons are participating in the National Childhood Encephalopathy Study. [Reproduced with permission of Professor David Miller, National Childhood Encephalopathy Study, Central Middlesex Hospital.]

ancies relate to differences in the record kept by the practitioner and the specialist in community medicine, in terms of 1) the date on which the vaccine was given, 2) whether it was diphtheria, pertussis and tetanus—or diphtheria and tetanus vaccine, and 3) the batch number.

Finally, if I may move away from this particular point of assessment of severe reactions, I would not agree that the present epidemic in the United Kingdom is mild, as I don't think you should assess the severity of pertussis on the number of deaths (i.e., on death-mortality rates). It is the length of time in the hospital which is so important. In my own personal view, we are experiencing quite a severe epidemic.

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Table 1. National Childhood Encephalopathy Study. Guidelines for Notification of Cases of Serious Neurological Disorders.

Notify All Cases with Confirmed or Possible Diagnosis of:	Do Not Notify:
1. Acute or subacute encephalitis/encephalopathy [a] Acute or subacute encephalomyelitis (Include cases of suspected viral origin)	1. Chronic encephalopathy
2. Unexplained loss of consciousness ± CSF or EEG changes.	2. Neurological complaints due to CONFIRMED toxic (chemical) cause or bacterial cause or metabolic cause or brain tumor or abscess or trauma
3. Convulsions complicated by one or more of: fits or series of fits lasting about ½ hour or more coma lasting 2 hours or more hemiplegia or other neurological signs not previously present, lasting 24 hours or more	3. Fits or series of fits lasting less than ½ hour with none of the complications listed opposite
4. Infantile spasms (West's syndrome) or Reye's syndrome [b] (cerebral degeneration and hepatomegaly)	4. Uncomplicated viral (aseptic) meningitis *without* encephalopathy

IF IN DOUBT PLEASE NOTIFY.

NOTE: IF THE CASE IS CONSIDERED A COMPLICATION OF VACCINATION PLEASE *ALSO* USE YELLOW CARD SYSTEM.

[a] The term "encephalitis" is taken to indicate any infective or inflammatory cerebral disorder. The more general term "encephalopathy" is used when the cause of the cerebral disorder is not immediately obvious.
The clinical features of both types of illness may include: altered level of consciousness; confusion; irritability; changes in behavior; screaming attacks; neck stiffness; convulsions; visual, auditory, and speech disturbances; motor and sensory deficit.
[b] Arch. Dis. Child. 48:411, 1973.

DR. GRIFFITH: Assessments of the effects of pertussis vaccination on pertussis mortality and morbidity rates in Britain tend to rely on an assumption that there was little or no pertussis vaccination before the inception of a mass campaign in 1957, after which a high percentage of infants or of the pertussis-susceptible section of the child population were vaccinated. But the vaccination program developed gradually from 1937, when the first immunization clinic was established, to 1960, when the last of the local health authorities instituted its vaccination arrangements (Table 3). Most local health authorities, particularly the large urban ones, had approved vaccination programs since the late 1940s or early 1950s superimposed on their efficiently organized diphtheria immunization programs. The Medical Research Council's report on poliomyelitis after childhood vaccination related to over 300,000 infants fully immunized against pertussis between May 1951 and December 1953 in large towns in England and Wales and manufacturers' records indicate that substantial amounts of vaccine were distributed at the time.

Another point I wish to make is that the term "mass vaccination campaign" is inappropriate when applied to the development of the pertussis vaccination program in Britain. The mass vaccination campaigns against diphtheria in 1942 and poliomyelitis in the late 1950s were directed at the entire disease-susceptible section of the population, namely, those under 15 and 40 years of age, respectively. But pertussis vaccination has always been restricted to infants under 1 year of age, and since one-third of notified cases are aged 2 to 3 years and another third, 4 to 9, there is a delay of a few years before the

benefits of widespread vaccination of infants under 1 year of age begin to affect notification. Thus, as shown in Figure 4, the widespread use of pertussis vaccines in England and Wales in the late 1940s and early 1950s appears to have been responsible for the sharp decline in notifications of disease during the late 1950s.

H.V. – Health Visitor
SCM – Specialist in Community Medicine
G.P. – General Practitioner
CC – Case control
ANO – Area Nursing Officer
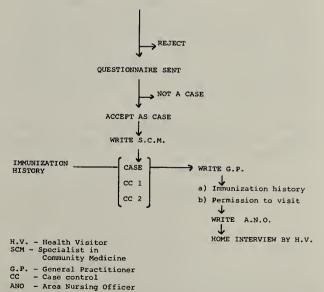
Figure 2. National Childhood Encephalopathy Study. Notified cases, aged 2 months to 3 years. [Reproduced with permission of Professor David Miller, National Childhood Encephalopathy Study, Central Middlesex Hospital.]

ALL CASES

RECOVERED BY 15 DAYS

4 - 6 WEEKS AFTER ADMISSION

WRITE G.P. AND CONS.

REPEAT FOLLOW-UP DETAILS

6 MONTHS AFTER ADMISSION

REPEAT FOLLOW-UP

RESIDUAL SIGNS

VISIT

a) Standard
   assessment
b) Home
   interview

REPEAT VISITS
at 6/12 + 12/12

SELECT 2 HOSPITAL CONTROLS
(with cons. permission)

as CC 1 + CC 2

G.P. - General Practitioner
Cons. - Consultant(s)
CC   - Case control
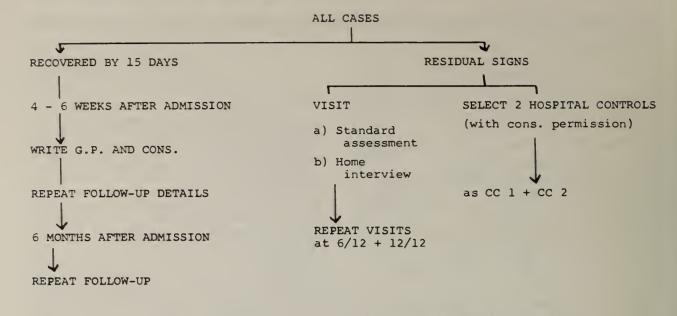
Figure 3. National Childhood Encephalopathy Study. [Reproduced with permission of Professor David Miller, National Childhood Encephalopathy Study, Central Middlesex Hospital.]

Publicity accorded in recent years to doubts over the safety and efficacy of pertussis vaccines has resulted in a sharp decline in the percentage of infants vaccinated (Figure 5). As a result there have been successive cohorts of mainly unvaccinated children reaching the ages of 2, 3, and 4 years, when they are particularly prone to contract whooping cough. This seems to account for the 1978 epidemic in England and Wales. As expected, the proportion of 2- and 3-year-old children among those reported is greater than in previous decades (Table 4).

DR. ZAKHAROVA: 1 would like to say a few words concerning the role of vaccination for control of pertussis.

According to the data of World Health Statistical Reports, annual reference books, and publications of epidemiologists of some countries, we have drawn up a review on pertussis morbidity and vaccination in developed and some developing countries for the last 25 years.

This analysis has clearly showed that after the introduction of pertussis vaccination the world distribution of whooping cough has changed significantly. The discrepancy in rates of morbidity and mortality among countries has become discernible. Thus, in 1975, in some countries the rates were 0.64–0.97 per 100,000 population; in several countries, 2.13–5.8 and 15.9–29.0; but in countries where vaccination has not been carried out, the rates were 366.2–608.0.

By the pattern of morbidity curves the countries can be divided into four groups. The first group includes countries in which the introduction of vaccination re-

Table 2. National Childhood Encephalopathy Study. History of Diphtheria/Tetanus/Pertussis and Polio Vaccination[a]

Past 0–7 Days	Immunized	Not Immunized	Total
Cases: No.	8	247	255
%	3.1	97	100
Controls: No.	12	484	496
%	2.4	98	100

[a] Immunization history of the index cases compared with the nonimmunized matched controls. This includes only children receiving DTP vaccine and OPV in the 7 days prior to admission. [Reproduced with permission of Professor David Miller, National Childhood Encephalopathy Study, Central Middlesex Hospital.]

Table 3. Expansion of Pertussis Immunization Program, England and Wales, 1950s

Year	No. of Local Authorities with Approved Schemes	No. of Children Completing Course
1951	76 (out of 129)	
1952	87	
1953	103	
1954	114	
1955	125	
1956	130	Provocation polio report
1957	136 (out of 146)	
1958	144	470,290
1959	144	527,945
1960	145	626,401
1961	145	690,070
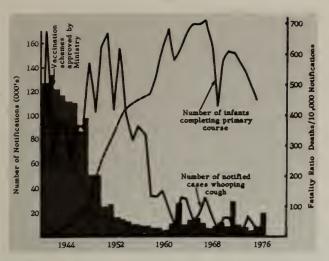
Figure 4. Whooping cough notifications, fatality ratio, and immunizations, 1940–1976.

Table 4. Age Distribution of Notified Cases of Whooping Cough, England and Wales

Age Group (years)	Percentage Distribution of Cases			
	1945	1957	1968	1978 (1st quarter)
0–	11.0	9.5	9.0	11.0
1–	28.5	20.0	24.0	15.0
3–	28.0	24.0	27.0	44.0
5–	28.0	41.0	34.0	24.0
10–	2.5	3.5	3.5	4.0
15–	0.5	0.5	1.0	2.0
25–	1.0	1.0	1.5	1.0
Unstated	0.5	0.5	0.5	—
Total notifications	62,663	89,017	33,531	15,654

sulted in a progressive decrease of whooping cough morbidity rates and in 1974–1975 they were 0.3 (Japan); 0.5 (Hungary); 0.86 (U.S.); 1.79 (C.S.S.R.); 2.5 (G.D.R.); 5.8 (U.S.S.R.) and so on.

The second group comprises countries in which the incidence of the disease dropped at the beginning, but then for a long while remained unchanged, and began to go down again from 1967–1969. In France, for instance, the morbidity rates ranged between 12.0–14.0; in Italy between 13.3–32.5 from 1950 to 1967. (Vaccination in these countries was started in 1954 and 1953, respectively.)

In the third group of countries, the pertussis morbidity rates became considerably lower after the introduction of vaccination; however, the decrease was cyclic with intermittent increases. For instance, in Bulgaria, where vaccination has been carried out for 18 years, the 1958 pertussis morbidity rate was 205.5; in 1967—87.9; in 1969—

9.0; in 1970—again 77.3; in 1972 and 1974—5.5 and 89.2, respectively.

In England and Wales, where vaccination has been carried out for 26 years, repeated rises were also observed. For instance, in 1962 the morbidity rates were 18.1, but in 1963 they became 77.3, in 1969–10.3, in 1970—34.2, in 1973—4.9, in 1974—32.9, and in 1975—15.9 (i.e., on the same level as in 1962).

At the same time in countries of the fourth group where vaccination has not been carried out (Congo, Senegal, and others), the morbidity rates were on the same high level as in countries from the first three groups before the vaccination was started.

Thus, together with high effectiveness of vaccination in countries of group I, there came to light obviously irrational application of use in other countries (groups II and III) and these results, evidently, have given rise to doubts of its effectiveness.

The importance of vaccination as a restricting factor of pertussis prevalence was particularly obvious in institutions of our country for pre-school children. For example, in the Estonian S.S.R., despite the significant increase in number of creches and kindergartens and of children in them, accompanied by the increase of contacts between them, pertussis morbidity did not rise. It occurred in only a small number of institutions. Thus, in 1958, whooping cough was reported in 57.7% of institutions for pre-school children; in 1974, only 2% of institutions reported pertussis and these were only individual cases (Table 5). Pertussis morbidity rates in children in pre-school institutions were 5–7 times lower than among other children. Before the introduction of vaccination preschool institutions always had significantly higher rates.

The ratio of morbidity of measles to whooping cough also supports this conclusion (Table 6). Both these diseases occupied by turns the first place among children's infections in the majority of countries before the introduction of pertussis vaccination. Pertussis morbidity rates in the Estonian S.S.R., although generally 1.5–6.3 times lower, were higher than those of measles in certain years. This relationship changed sharply after the introduction of vaccination against whooping cough. The same

Figure 5. Live births, whooping cough notifications, and pertussis vaccinations, England, 1967–1978.

Table 5. Pertussis in Preschool Children Institutions in Estonian S.S.R.

Year	Increase in number (x)		Morbidity Rate per 100,000	Institutions in Republics with Pertussis (%)
	Kinder-gartens	Creches		
1958	1	1	583.0	57.7
1966	2	3.5	42.0	
1972	4.6	10	5.4	
1975			2.3	2

situation was observed when compared with scarlet fever. The ratio of scarlet fever to pertussis morbidity rates was 1954—1; in 1955—0.54; in 1967—29.3; in 1972—40 and in 1974—52.5.

I believe that social, demographic, and medical developments in a country cannot have a major influence on the decline of pertussis disease. This is demonstrated once more by the present situation in Japan, where cessation of vaccination has resulted in a sharp rise in the incidence of whooping cough; reported cases of whooping cough increased from 206 cases in 1971 to 2,508 in 1975.

DR. FOX: Two or three points that were made verbally this morning are worthy of comment. Dr. Stewart stresses the relationship between environmental factors and the occurrence of disease. He neglects to point out, of course, that there is also a similar parallelism between acceptance of vaccine and the same social environmental factors.

Second, he talked about the high proportion of cases that have had a history of vaccination. He does not attempt to estimate the efficacy of vaccine with these data because he has not used any denominators. I understand that in earlier publications he has shown that the vaccine was at least 80 percent effective. I think the point of efficacy of the vaccine is the central issue here. Furthermore, Dr. Stewart in his published work and the manuscript submitted for this symposium mentioned 197 children who had brain damage. He says these all had severe and gross brain damage.

Evidence of this was not presented here today. I have no idea over what time period these data were collected,

Table 6. Pertussis and Measles in the Same Countries, 1973

Country	Morbidity Rate per 100,000	Number of Cases		Ratio M/P
		Pertussis(P)	Measles(M)	
Japan	0.39	364	22,418	61.6
Hungary	1.5	155	53,826	347
ChSSR	2.4	345	19,858	57.5
Argentina	127.6	29,604	20,727	0.7
Mexico	29.5	14,986	13,734	1.0
Congo	502.9	6,538	9,038	1.4

but I think the important point is that these cases were examined by him because they had received vaccine. I would like to know whether he examined cases that might be identical, but did not receive vaccine. Obviously, the prospective study that Dr. Dudgeon described is going to provide the right answer. The kind of study that Dr. Hannik from the Netherlands described sets a sort of upper limit on significant brain damage, and seems to be in great conflict with the figures or the estimates that Dr. Stewart has arrived at. I am much more convinced by her upper limit figures than I am by the vague estimate figures that Dr. Stewart presented.

DR. STEWART: As far as the acceptance rate of the vaccine is concerned, we dealt with this in an earlier publication to which reference is made in the text of the paper. The acceptance rate of vaccines, of course, has gone down. It is now about 35 percent, whereas it used to be about 65 or 70 percent, but we did show in a previous publication that there was—and I agree with Dr. Stuart-Harris almost exactly in terms of the correlation coefficient that we got—there was an inverse correlation between acceptance rate of vaccine and attack rate of whooping cough. Nevertheless, when one did a multivariant analysis—and this is detailed in the publications—the effect of the acceptance rate was lost, but the effect of the socioeconomic factors was retained.

The paper is full of denominators: 1) the population at risk in Glasgow and the number of cases there, 2) the vaccine-damaged children, the number of children vaccinated, and the number of cases reported to the Committee on the Safety of Medicines. In all cases, the estimates of incidence are based on those denominators.

What is not, perhaps, too explicit is that there is considerable underreporting of vaccine damage to the Committee on the Safety of Medicines, and there is a representative here of the DHSS in Britain, who would agree that the figures do not represent more than 10 percent of the total.

Finally, with regard to brain damage, none of these cases was evaluated by me as being brain damaged. All were diagnosed in various hospitals in the United Kingdom, many of them in the Hospital for Sick Children at Great Ormond Street, London.

DR. HENNESSEN: The interpretation of the graph we saw of the city of Hamburg deserves some explanation. It has been said that the vaccine was withdrawn from Hamburg. It has never been withdrawn because it has never been used in public health service, but it has been and is still being used by private practitioners.

The second point I want to make about Hamburg is that Hamburg lost one-quarter of its resident population during the last 15 years. Hamburg is a city-state, as we call it, and the residents moved into the country, and in doing that, of course, they thinned out the juvenile population, and therefore, the exposure is very small. For this reason, Hamburg cannot serve as any convincing evidence pro or contra vaccination.

DR. COHEN: Professor Hennessen, are there reliable data about the morbidity of pertussis in Germany?

DR. HENNESSEN: Pertussis is not notifiable in Germany.

DR. J. B. ROBBINS: I would like to emphasize one point about the diagnosis of the so-called pertussis encephalop-

athy. This is not a distinct clinical syndrome, and there are no pathonomonic, pathologic findings which would allow a clinical doctor or pathologist to assign the diagnosis of pertussis encephalopathy. Rather, the diagnosis can only be made by association with vaccine usage and exclusion of other diseases known to cause brain damage. It is a very poorly defined clinical disease.

DR. CAMERON: Dr. Stewart said in his abstract that assessments made in 1968 showed that vaccines used during the preceding decade in the United Kingdom were largely ineffective. During the period 1958 to 1972, I was responsible for the production of pertussis vaccine at Burroughs-Wellcome and, over that period of time, I can assure my colleagues that there were no substantive changes whatsoever in manufacture in terms of quality or anything else of B–W vaccine. I have also said repeatedly at meetings that pertussis vaccine is not an entity like sodium chloride. It cannot be nearly so well defined, and I think we should consider the possibility of differences from one manufacturer to another.

It is much easier to pose questions in pertussis work than to answer them. Nevertheless, I think the questions have to be posed, and I think we have to try to seek answers.

Over the period I am talking about in the early 1970s, the depressing comments on British vaccines were followed by more reassuring comments, particularly in the classical paper by Preston which described "brighter horizons."

DR. STEWART: I entirely agree with Dr. Cameron's statement. I know that the B–W vaccine was unchanged over that period. This is very interesting because it was during the period of 1958 to 1968 that those vaccines were deemed by the Public Health Laboratory Service to be ineffective.

These were the vaccines which, as Dr. Cameron has said, were unchanged. Now there are views about the relative role of agglutinogens, but I think that there is a considerable support for the point of view that any change in the agglutinogens and agglutinogen content did not alter the efficacy or otherwise of the vaccines.

DR. STUART-HARRIS: May I make just a brief comment on the change in the vaccine? It was not, I think in 1968 that this change occurred; it was about 1964. It was done so because, in fact, the actual bacilli circulating were found to contain the 1.3 antigens, whereas the vaccine itself had a low component of 1.3. It was also brought up to strength according to the international units.

The vaccine has been examined in a study by the Public Health Laboratory Service and found to be less effective than the estimates in the Medical Research Council's trials, but it would be wrong to say it was ineffective.

May I make a second comment on behalf of the Department of Health in Great Britain, because the comments that have been made from Professor Stewart are data from the Committee on the Safety of Medicines. These were shown to him. They are not, in fact, publishable because they belong to the Committee. They are, therefore, confidential to the individuals concerned. They are not data which can be used to estimate risks and

this has never been considered. They are merely pointers to hazards.

The figure of 10% reporting referred to by Dr. Stewart was obtained in another study of reactions to drugs, which was deliberately carried out in order to try to ascertain the percentage of actual reactions that were being reported.

We do not know the rate of reactions reported in relation to vaccines. This may be found, I suppose, by some later survey, but as we estimate the rate of notifications against infectious disease, it is not better than 30 percent. It would not be surprising if the rate of adverse reactions to anything was higher than 30 percent.

DR. CAMERON: Just a supplementary remark, in a sense, to both Sir Charles and to Dr. Stewart. Burroughs-Wellcome vaccine, fortuitously I confess, during this whole period contained a total complement of the three agglutinogens, and this is on record both at Burroughs-Wellcome and with Dr. Noel Preston.

Second, I myself was deeply involved in the question of potency standards. Burroughs-Wellcome vaccine always contained four protective units per dose regardless of the shortcomings of the British standard, because we were selling vaccine both in the United Kingdom and abroad, and it was somewhat invidious to suggest that vaccine being sold abroad should contain 4 protective units and vaccine on the British market 2.1 protective units, which was the British standard. This period during which there was constancy of composition did not really stop at 1968. It went on to 1972 over the period of time when we were describing brighter horizons. From 1958 to 1972 no changes were made in the preparation of the vaccine; since 1958 it has contained both four protective units per dose as a minimum, and a full complement of three agglutinogens considered to be important.

DR. CSIZER: I would like to make a short statistical comment on the paper of Professor Stewart. He showed a slide indicating that before the vaccination era, the death rate decreased more steeply than afterward. But he used linear regression analysis instead of nonlinear, which would have been more correct.

DR. J. W. G. SMITH: Dr. Hannik presented a most interesting study, but I am not clear whether the reaction rate she describes is in terms of vaccinated babies or per dose of vaccine.

Second, would Dr. Hannik describe what she meant by shock?

DR. HANNIK: I don't estimate the role of reactions per injection. If you observe a reaction in a child, you stop vaccination. We have seen recurrences in the same child because the parents did not warn the doctor after the first injection. I therefore never give a rate per injection, but always per total number of vaccinated children.

As regards your second question, shock is an alarming situation. The child turns suddenly ashen gray, a marblelike color, and loses consciousness. The diagnosis is made afterwards on evidence from the mother. There are therefore two criteria, the description of the color and a feeling of the mother that her child was dying. Every child I have on the list met those two criteria. All recovered without therapy.

DR. SMITH: Do they scream?

DR. HANNIK: No.

DR. BLASKETT: Sir Charles Stuart-Harris mentioned as an aside, that they expected a respite from pertussis during the summer season. Professor Stewart has also indicated the effect of environment on the incidence of pertussis.

If you read a textbook written in Europe, you will probably discover that whooping cough is an autumn-winter disease of children. If you look at Table 7, you will discover that whooping cough caused by *B. pertusiss* is predominantly a spring-summer disease in Australia and is, in fact, *least* prevalent there in winter. Interestingly, the incidence of type 1.2 infections is disproportionately higher here in autumn and winter than it is in spring and summer. This difference is actually statistically significant (p = 4%) and so is presumably real.

I would like to know whether, in this new outbreak of pertussis in England, the same serotype is isolated, because we have a change in Australia.

DR. STUART-HARRIS: Type 1.3 has been current throughout the epidemic, and your remark about the prevalence of pertussis in the summer in Australia is much appreciated. We always appreciate that things are upside down in your part of the world, but we do have summer holidays, and that is really why we expected a little respite.

DR. STAINER: I am sure it is very interesting for Professor Stewart to quote multivariant analysis and all the other statistical aids to show why pertussis vaccination doesn't appear to be working, but to a mother of a child suffering from pertussis in Britain (where I believe 32,000 cases have been reported this year), it would be of very little use or satisfaction!

I think one has to be realistic. Pertussis vaccine has been reasonably successful over the years. The recent campaign mounted against vaccination by certain people (who had limited facts to go on) led to unfavorable publicity and vaccination came into disrepute.

Vaccine damage is something that will always concern anyone who is involved in the manufacture and control of pertussis vaccine. What we have seen during this meeting permits us to define certain things about pertussis: everybody knows that pertussis vaccine is pyrogenic, resulting in a fever in children and animals. Can we produce a pertussis vaccine which is nonpyrogenic? Which of the many factors that we have heard about could we remove while maintaining protection? Pros-

pective studies on reactions, such as those outlined by several workers, should do much to allay the fears of the anti-vaccination groups and lead to a scientific confirmation of the value of pertussis immunization.

DR. J. B. ROBBINS: I would like to ask the first two speakers, as well as the Danish group that is here, if they have tried to calculate the age-specific attack rate at the next 4-5 year interval if the trend for decreasing vaccine usage continues? Have they projected what they should anticipate based upon the cyclic increase of the disease and the observed increase in relation to decreased vaccine usage?

DR. KATZ: Just as Dr. Hannik was asked the question about the shock syndrome, I was quite surprised by Dr. Baraff's and Dr. Cherry's data on what they called "screaming." But I was reassured when I heard the tape they played, because from 22 years' experience in pediatrics I would think this was crying. The difference between crying and screaming may be a matter of semantics, but I think those of us who have dealt with children and especially neurologically damaged children interpret a scream as something quite different: high-pitched, shrill, piercing. What we are used to hearing is the scream of the child with encephalitis, the child with bilirubin encephalopathy, the child with intracranial hemorrhage. What I heard from the tape was not what I would define as a scream. I wondered if this was due to the equipment that was used, or was that indeed representative of what Drs. Baraff and Cherry are calling screams? Because I would dispute the use of the word then and say that that is prolonged crying, but not screaming.

DR. BARAFF: I agree completely that we should change the term to prolonged crying. I also think that this sounds like a child crying, and we played this tape along with some controls that we have recorded, and people were unable to tell crying from this type of reaction.

DR. DUDGEON: Unfortunately, I don't have my tape in my pocket, but I didn't think that this was a cerebral cry or scream. It was quite a different sound from the very few that I have heard which Dr. Katz describes as a very high-pitched noise.

DR. RELYVELD: I would like to ask Dr. Stewart a question about one of the slides he showed. I saw a comparable slide in June in Sweden. It concerned tetanus vaccination and local reactions and the slide reported, let's say, 50 children with pain in their arms, 40 of whom were crying, so there were probably 10 of them who were very courageous. It was indicated that there were 90 reactions. Now a child in pain will cry and there is no reason to separate these two observations. If, however, you add convulsions, crying, shock, and so on, you may come to a wrong addition if they are manifestations of the same reaction.

DR. STEWART: I did not show a slide relating to pain, and especially not in tetanus, and certainly not in Sweden.

DR. RELYVELD: I want to state that as recently published, the Pasteur Institute distributed 720,000 doses of pertussis vaccine, combined or single, in 1976 and 1977 and that during that time no neurological reactions were reported to us. I think that the reason is that these

Table 7. Seasonal Distribution of *B. pertussis* Isolates in Australia, 1950–1973

| Season | Serotypes | | | Totals |
	1.2 (%)	1.2.3 (%)	1.3 (%)	
Spring	27	41	46	143
Summer	41	43	38	137
Autumn	17	10	10	37
Winter	15	6	6	26
Totals	41 (100%)	80 (100%)	211 (100%)	343

vaccines are highly adsorbed and therefore less toxic. During the 1976–77 period, two deaths were reported. It was only possible to analyze one of these cases, and death after vaccination was due to acute *N. meningitidis* type B meningitis and not to pertussis vaccination as could have been claimed.

DR. COOK: A small point for the record. Dr. Stewart showed a slide that said pertussis vaccination began in Egypt in 1975. I can assure you that triple vaccine has been used with a high level of coverage in Egypt since the late 1960s. If anyone cares to write to me in Alexandria, I will send him the official figures of coverage of triple vaccine for the last 10 years. So whatever conclusion was drawn from the slide was not correct.

DR. STEWART: The data were obtained by a colleague in Cairo, and I understand that those are the official figures of notifications and deaths in Cairo, where the use of the vaccine in the public sector, so I understand, began in 1975.

DR. CAMERON: Could I just have a moment to reinforce what Dr. Cook said? I think those of us today who are indulging in the luxury of this argument about reactions versus efficacy ought to appreciate that the argument is wholly irrelevant in developing countries. When you have had the experience, as I have, of finding that the dose (of vaccine) has been reduced by half to prevent reactions when people were dealing with a disease with a 25 percent mortality rate, you realize the heavy responsibility that lies on our shoulders to see that this kind of misinformation does not get into areas where it can be so badly and dangerously misinterpreted.

DR. PICHICHERO: I would like to comment on Dr. Baraff's report. Dr. Roger Barkin of the University of Colorado in Denver and I were also interested in this question of reactogenicity to pertussis vaccine. In a preliminary study we attempted to gain an overview of this problem. (Barkin, R. M. and Pichichero, M. E. Diphtheria-pertussis-tetanus vaccine: reactogenicity of commercial products. Pediatrics, in press). During an 8-month period beginning in July 1977, we prospectively surveyed patients from four pediatric practices in Denver regarding reactogenicity following DTP vaccine administration with the use of a self-administered mail-in questionnaire.

All socioeconomic status groups were represented. Vaccine was administered according to current recommendations, and the children were healthy at the time of vaccine administration. Using the same parameters as Dr. Baraff—fever, behavior, and local reactions—we evaluated 1,232 vaccinations. We found that 54% of our patients had a temperature greater than or equal to 100°F rectally, and 4% of the children had a temperature greater than 102°F rectally. Significantly greater febrile reactions were seen with the first booster as compared to other vaccinations. This rate of fever response was observed, despite the use of antipyretics in 65% of the children.

Regarding behavior changes, 52% of our patients had either no behavior change or only mild irritability. Thirty-five percent had periods of unexplained excess of crying, and 13% had prolonged periods of screaming. There was a high percentage of local reactions.

Seventy-two percent had either redness, swelling, or tenderness at the injection site, but the degree or extent of these local reactions was not quantitated. However, local reactions were of greatest concern to the parents. A significant increase in local reactions was found with increasing age of the patients. No seizures, encephalitis, shocklike states, or hospitalizations were reported during the prospective evaluation of these vaccinees.

There was no significant difference in the reactogenicity rates among the children in the four practices, nor among those immunized with any of the three lots from one manufacturer and two lots of a second manufacturer. We were unable to develop such an elegant control population as Dr. Baraff and Dr. Cherry; however, we were able to obtain similar toxicity information from 57 patients to determine the incidence of fever and atypical behavior during the 48-hour period prior to a well baby checkup and vaccination with DTP vaccine and again in a 48-hour period at least 14 days after immunization. There proved to be no difference in the rate of symptoms and signs during these two control time periods. Interestingly 10–14% of the children had fevers of 100 to 102°F rectally in the two time periods, respectively, though no child had a fever of greater than 102°F. Approximately 15% of the children were considered atypically irritable in the control periods, but no child had bouts of crying or screaming.

Our preliminary overview study in Denver agrees substantially with that of Dr. Baraff and Dr. Cherry, and indicates that the current pertussis vaccine has substantial reactogenicity.

DR. FRASER: I have a question for Dr. Hannik. Was there any correlation between the occurrence of elevated temperature and the occurrence of elevated insulin levels in your patients?

DR. HANNIK: There was no correlation.

DR. GRIFFITH: I am rather concerned that the assessment in Dr. Hopkins' paper was of different vaccines at different clinics on different days. If vaccines are compared for early reactions, this should be done in the same clinic; the vaccines should be distributed randomly among the children, while the assessment should be blind. If you have one batch of vaccine used on different days and in different clinics, you will then get a wide distribution in results.

DR. J. W. G. SMITH: Would you allow me to make a comment on the papers that have reported reaction rates? It is difficult to criticize them without seeing the published data, but I think one has to interpret with great caution reaction rates culled from questionnaires comparing one vaccine with a rather similar one.

I have some experience in that sort of study with, for example, influenza vaccine, and if you ask leading questions of patients or the mothers of children, you get extremely high reaction rates. But if you follow patients up and look for clinically significant reactions, you get much lower rates.

DR. MANCLARK: During the final editing of Dr. Stewart's manuscript it became apparent that several items and issues contained in the text should receive some attention and clarification. One issue is contained in Sir

Eric Scowen's letter to me dated November 10. Sir Eric's letter and Dr. Stewart's response to it follow.

November 10, 1978

Dear Dr. Manclark:

In a recent International Symposium on Pertussis held in Washington on 1–3 November 1978, Professor Gordon Stewart of Glasgow quoted certain figures from the Committee on Safety of Medicine's Adverse Reaction Register. On behalf of my Committee I should like to see the following comment published in the proceedings of the Symposium:

> The Committee on Safety of Medicines issues the following written advice to all authors who wish to quote from the Committee's figures for adverse reactions to drugs: *on no account should the information be used to estimate the incidence of adverse reactions.* This advice is given for the following reasons:
>
> 1. The reports are not all confirmed or investigated at the time of receipt, hence knowledge of other medications, disease or disorder, and of temporal relationship with drug administration is lacking.
> 2. Reports may be of past events but are entered at the date of receipt.
> 3. A single report may recount several events, each of which may appear as separate entries in some circumstances.
> 4. The extent of reporting adverse reactions to the Committee is far from complete.
>
> Where an author quotes Committee on Safety of Medicine [C.S.M.] data in a publication, the Committee wishes it to be clearly understood that any interpretation or calculation based upon these figures is the author's own responsibility and not that of the Committee.

Professor Stewart was informed of these provisos. Thank you for giving us the opportunity of incorporating a statement on the misuse of our data.

Yours sincerely,
/s/
Sir Eric Scowen, Chairman
Committee on Safety of Medicines

December 13, 1978

Dear Dr. Manclark,

Thank you for sending Sir Eric Scowen's letter of 10th November. Here are the answers to his questions:

1. If Sir Eric Scowen will indicate which of the reported reactions are unconfirmed or not investigated, I shall amend the table [Table 9]. In the absence of such information the table stands as an accurate version of information officially received and reported by his committee.
2. Unless reports are delayed into the next calendar year, this objection will not alter the figures in the table.

3. I find it hard to believe that an expert medical panel would record two different reactions in different children as a "single report," but if they do, the figures in the table will be an underestimate.
4. Agreed. This is stated in the text and is one of the reasons for my concern.

Sir Eric Scowen and his secretariat were given a preview of the relevant parts of my paper on the 28th September 1978, and Table A was compiled from up-to-date information provided by them. I share their reservations concerning "estimates" of incidence but the inaccuracy in this respect is no different from notifications of infections [sic] diseases, sickness certificates, death certificates and other voluntary notifications, none of which are confirmed but are usually acceptable as *estimates* of incidence. The Committee on the Safety of Medicines is in fact required under the Medicines Act of 1968 [Section 5(2)] to receive and investigate adverse reactions reported to them confidentially by registered Medical Practitioners. I presume that Sir Eric Scowen is not, in his letter, suggesting that the figures thus obtained are meaningless.

Yours sincerely,
/s/
Professor Gordon T. Stewart

The second issue concerns some of the details of Dr. Stewart's manuscript. The following comments are the result of my review but represent a consensus following discussions with my colleagues. Doctor Stewart's concepts of pertussis vaccine usage are most important because they may affect pertussis vaccine policy and research for many years to come. It is not my intent to impugn Dr. Stewart, but it necessarily follows that arguments to support such a change in policy should be lucid, cogent, and properly presented. The following comments were contained in my letter of December 1 to Dr. Stewart. Doctor Stewart's responses were part of his letter of December 13. [Items are not completely quoted in order to conserve space, but are identified by page and line number.]

Page 263, lines 12–18(L) re Figure 1; also Figure 1 per se.
COMMENT: There is no description of the survey. How was the survey conducted, and by whom? No description of the selection and composition of the sample (including residence) surveyed.
RESPONSE: The survey is that of the Research Unit of the Royal College of General Practitioners as published in the given reference (99). The figures are derived from returns submitted by 40 general practices covering a population of approximately 200,000.

Page 263, lines 9–11(R): ". . .the striking rise that occurred during the years 1940–1945 . . ."
COMMENT: In Figure 2 the striking rise occurs in the 1945 to 1952 or 1954 period.
RESPONSE: The rise in 1945–1952 occurred in proportion to the rise in the postwar birth rate. The rise in 1941 occurred through a falling birth rate.

Page 263, lines 11–17(R) re Figure 2; also Figure 2 per se.
COMMENT: The regression line averages in the "abnormal" post-war period.
RESPONSE: The regression line applies as shown to the period 1940–1972.

Page 263, lines 26–29(R): ". . .per 1000 cases . . ."
COMMENT: Does Dr. Stewart have information on the age and vaccine status of cases and deaths?
RESPONSE: Unfortunately not. Does anyone have such detailed information for any country in 1950?

Page 264, lines 5–11(R): ". . .there had been no dislocation or herding of communities through bombing and evacuation . . ."
COMMENT: True, but the dislocation and herding occurred before the great increase in notifications. See Figure 2.
RESPONSE: 1939–1940 were the years of evacuation and dislocation; 1941, the year of bombing and herding in the major cities of the United Kingdom to which these figures refer.

Figure 3, page 264.
COMMENT: *What is the source of the data?*
RESPONSE: Registrar General, England and Wales, 1919–1977

Page 264, lines 5–11(R): ". . .a vaccine program had been adopted on a fairly wide scale [in the United States and Canada] by 1950." Also Figure 4.
COMMENT: The Old World and New World are not so different on this count. Data were presented during the discussion period of Session 5 that a high proportion of children in England and Wales were vaccinated with good vaccines starting in 1950.
RESPONSE: Disagree. Vaccine usage in the United Kingdom was irregular and highly incomplete until 1957, except in a few centers where trials were proceeding and to which I have referred (pp. 264–265). The data presented in the discussion period of Session 5 came from a representative of a pharmaceutical company and related only to their sales to unspecified buyers. There are no official records prior to 1957 of which vaccines were used, how tested, how and when administered.

Page 264, lines 22–26(R): ". . .nor did it show that an epidemic as opposed to a sporadic outbreak could be controlled."
COMMENT: Few, if any, clinical trials provide evidence that an epidemic can be controlled—they required 1) efficacy of vaccine, and 2) widespread acceptance of vaccine in susceptible population groups.
RESPONSE: Agreed. This is another way of saying that this vaccine is relatively ineffective.

Page 264, lines 37–43(R): ". . .on this basis, which by present standards would be unacceptable in the United Kingdom and the United States . . ."
COMMENT: On what basis? It is not clear what is meant

by present standards and why and how they are unacceptable.
RESPONSE: Present standards require adequate, controlled trials and absence or explanation of toxicity before a product is licensed. Informed consent is also required. A trial based upon control infants injected with a placebo of various killed bacteria and their products as in the M.R.C. trial (7,8) would not be either ethical or legal.

Page 264, lines 44–46(R): ". . .the vaccines used before 1968 were practically ineffective."
COMMENT: Does this mean vaccines used prior to 1968 were ineffective, or does this refer to a particular period of time that ended in 1968?
RESPONSE: The summary of the final report of the P.H.L.S. Committee and Working Party reads ". . . among vaccinated contacts under 5 years in homes in which *B. pertussis* was isolated, 52% developed paroxysmal cough. The corresponding attack rate in unvaccinated contacts was 69%. These findings suggest that much of the pertussis vaccine in use for 5 or 6 years before 1968 was not very effective . . ." In reference 1 there is also a comment on the ineffectiveness of vaccines used before 1968.

Page 264, lines 50–51(R)—page 265, lines 1–6(L): ". . .the sting went out of the disease . . .the vaccines were manifestly ineffective in controlling the epidemics of 1963–64 and 1967–1968."
COMMENT: This is not justified by the data. 1) Diminished sting could well reflect modification of the disease that did occur despite vaccine; 2) 1963–1964 and 1967–1968 were minor cyclic variations rather than epidemics; 3) "control" requires a high level of acceptance as well as efficacy.
RESPONSE: 1) Total mortality decreased from 650 in 1929 to 25 per million in 1950; and case mortality, proportionally. Complications (26) were uncommon from 1945 onward. To anyone seeing children thereafter, it was obvious that the disease was becoming much less severe. 2) If the epidemics of 1963–1964 and 1967–1968 in the United Kingdom were "only minor cyclic variations," then so is the present "epidemic." 3) Can the reviewer prove this?

Page 265, lines 32–35(L): ". . .there had been no substantial fall in vaccine-acceptance for the child population (Fig. 2)."
COMMENT: How is this shown in Figure 2?
RESPONSE: Figure 2 shows the number of children vaccinated as computed from figures published by the D.H.S.S. (4,16,99).

Page 265, lines 35–36(L): ". . .the outbreak of 1970–1971 was not prevented."
COMMENT: As stated previously, control requires a high level of acceptance as well as efficacy.
RESPONSE: How high? Acceptance in infants in 1963–1973 was generally above 70%, often over 80%.

Page 265, lines 43–46(L): "There were 41 deaths, mainly in children in the 0–2 year age group, of

whom about 1.25 million (about 80%) had been fully vaccinated during the preceding 2 years."

COMMENT: Possibly true, but what were the death rates in the vaccinated versus the unvaccinated?

RESPONSE: See Table 2. Thirty-five of the deaths were in the 0–1 age group, mainly below 9 months of age, and therefore in the United Kingdom at that time below the age of completion of vaccination.

Page 265, lines 26–27(R): ". . .had severe congenital lesions."

COMMENT: How does this relate to the argument?

RESPONSE: Vitally. A child with a severe congenital lesion —such as a congenital cardiac anomaly—is vulnerable to any respiratory infection, and indeed to any mishap.

Page 266, lines 11–17(R): ". . .a substantial proportion . . .younger preschool children," also Tables 3 and 4, page 267.

COMMENT: Not age-specific. Rates in vaccinated and unvaccinated are needed. What is lacking is: 1) age breakdown, i.e., <1, 1–4, 5–9, etc., and 2) corresponding denominators for each category of vaccine status.

RESPONSE: I welcome this suggestion, which should be applied also to the papers by Linnemann, Broome, Mortimer, Stuart-Harris, Hannik, and several other speakers. With the outbreak in Glasgow still in progress, I could not complete this part of my study because many children in homes that we visited might have been incubating the disease. My fuller results will be published in due course, but meanwhile the statement stands: of 609 children notified as whooping cough, 34% are known by inspection of health records to have been vaccinated thrice with DTP, i.e., fully vaccinated on U.K. schedules.

Page 267, lines 1–4(L) and Table 5: ". . .found repeatedly that 40% or more of cases of whooping cough occur in fully vaccinated children . . ."

COMMENT: The fact that 40% of cases occur in fully vaccinated children (if true) has only one meaning: that vaccination is not 100% effective. Consider the following:

1. No one in the population has been vaccinated; thus 0% of cases occur in vaccinated persons.
2. Everyone in the population has been vaccinated: thus 100% of any cases that do occur were in the vaccinated persons.

One must admit that no vaccine is 100% effective. The question is "how effective"? Without attack rates (age specific) in vaccinated and in unvaccinated, the question cannot be answered. Surely Dr. Stewart knows this and would want to correct this misleading statement.

RESPONSE: Age-specific rates will of course be helpful, but nevertheless the statement as it stands is true. If a disease occurs in vaccinated children, the efficacy of the vaccine is, to say the least, questionable. Why not apply this criticism to those who think that the vaccine is sufficiently effective to be mandatory for every child?

Page 267, lines 7–19(R): ". . .cluster . . ."

COMMENT: What is a cluster?

RESPONSE: A "cluster" is a "number of things of the same kind . . ." i.e., exactly as used in the text to describe certain areas with common demographic features.

Page 268, lines 1–4(L): ". . . a schoolgirl of 15, fully vaccinated . . ."

COMMENT: When was she fully vaccinated?

RESPONSE: In infancy, recommended in the United Kingdom by the D.H.S.S. and manufacturers.

Page 268, lines 22–26(L): ". . .the secondary attack rate . . . and unvaccinated contacts."

COMMENT: Again this needs to be documented with regard to 1) age-specific rates and 2) some index of relative severity.

RESPONSE: It does not "need to be documented." As a statement of opinion based on fact, already documented and generally agreed (5,7–9,18,19,20,23,28,43), it is correct.

Page 269, lines 23–27(L): ". . .pertussis as a relatively mild infection . . ."

COMMENT: What is the basis for thinking of pertussis as a relatively mild infection?

RESPONSE: There have been no deaths in Scotland since 1974, none in Glasgow since 1970, and very few in the United Kingdom. Complications are also rare (18,23,26,28). The disease is undoubtedly distressing in young children and uncomfortable at all ages, but it is not nearly so bad as it was 30 years ago.

Page 271, lines 19–21(L): "By definition a detoxified product is likely to lose mouse-virulence and disqualify itself for further testing."

COMMENT: This statement is not clear and is not based on scientific fact or actual practice.

RESPONSE: Neither is the mouse protection test.

Table 7, page 268.

COMMENT: Data on age of children and severity of disease are needed.

REPONSE: Call this table incomplete, and I shall agree. But it is a statement of fact, nevertheless, and is admissible as such. The data are Dr Ditchburn's and have been submited in full to the *Lancet* for publication.

Table 9.

COMMENT: This table is not self-explanatory and is not helped by relevant text (page 272). Also, see comments in Sir Eric Scowen's letter.

RESPONSE: See answer to Sir Eric Scowen's letter.

Table 10.

COMMENT: What is the time frame for brain damage following DTP immunization? Also, neither in the table nor in the relevant text (page 272) is the basis for separating out 350, 500, 750, etc. indicated. It should be noted that until comparable data in control (not recently vaccinated) populations are produced, these data have little meaning.

RESPONSE: Reactions, as described after DTP (screaming, convulsions, etc.), usually [occur] within 24 hours. The numbers (250, etc.) refer to the numbers reported to the C.S.M. over the period 1957–1976. What does the reviewer mean by such data having "little meaning"? If a well child has a fit and becomes mentally defective after a shot of vaccine, does that have "little meaning" unless all vaccinated children react in that way, and all unvaccinated children do not?

Table 12, page 273.
COMMENT: Source of data and time of occurrence not stated.
RESPONSE: [None]

Tables 15 and 16, page 275.
COMMENT: What are the bases for the consultant's opinions?
RESPONSE: Documentation (on file).

Page 274, lines 40–41(L)—1(R): ". . .investigated in detail . . ."
COMMENT: Investigated by whom?
RESPONSE: By myself and the C.S.M. Secretariat (not all cases as yet). Full details of all cases are on file.

Page 274, lines 6–9(R): ". . . arrest of development . . ."
COMMENT: How can "arrest of development" be observed within 24 hours?
RESPONSE: If a previously well and responsive child fails to recognize its mother or to respond to stimuli.

Page 274, lines 14–17(R): ". . . attributed . . ."
COMMENT: On what bases were the reactions and sequelae attributed to pertussis vaccine?

RESPONSE: Ask the pediatricians concerned.

Page 275, lines 16–18(L): "All of the children . . . handicap or both."
COMMENT: The circumstances relating to the 197 cases of brain-damaged children who are the subjects of Tables 11–16 are never stated. Somehow, they emerged from the yellow card series. But 1) the basis for judging them brain-damaged is not stated; 2) by definition (and intent) they all were in vaccinated children, so that 3) without information about similar cases in unvaccinated children and hence some estimate of attributable risk, all of this is essentially meaningless, as Dr. Stewart himself so states.
RESPONSE: 1) They were all diagnosed as brain damaged by pediatricians in hospitals. 2) Agreed. 3) Nonsense. See comment on Table 10. Interpretation is often difficult and requires caution. If the reviewers' criteria had been applied to thalidomide and chloramphenicol, we would have continued to use them freely until a controlled trial had been completed.

Page 276, lines 4–7(L): ". . . a disease that seldom nowadays threatens the life or health of well children."
COMMENT: I think that Dr. Stewart would agree that pertussis is a major disease problem in the under-developed countries (cf. Dr. Cook's presentation in this symposium).
RESPONSE: In some developing countries. Dr. Cook's study related only to Egypt where, as it happens, my colleagues have data suggesting that whooping cough has decreased there in incidence and severity without any extensive use of vaccine.
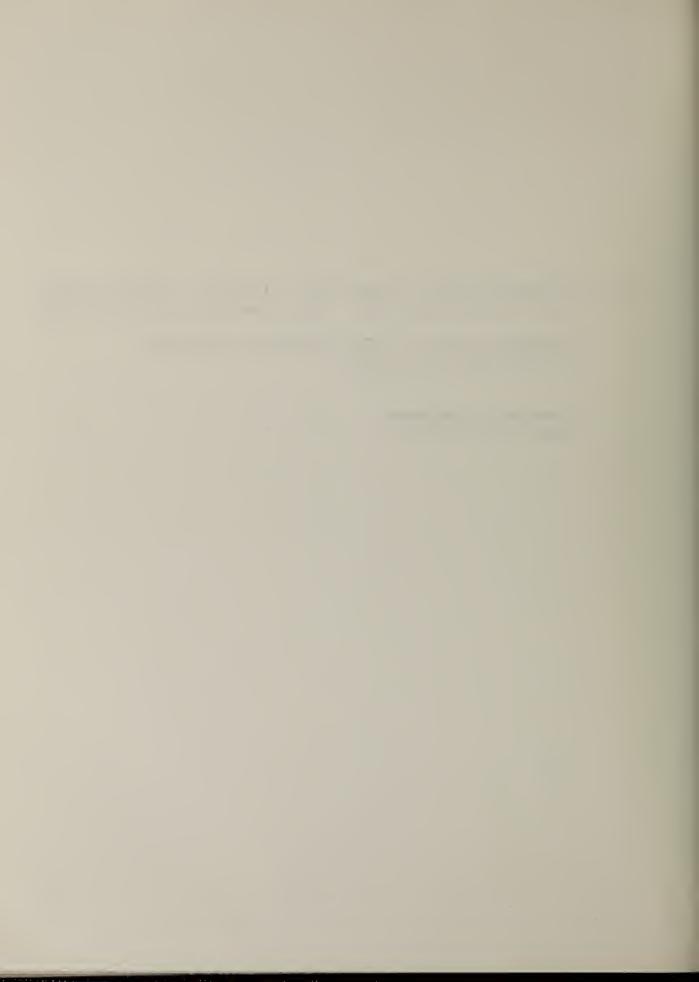
Part 6. PROSPECTS FOR IMPROVED VACCINES AND METHODS
FOR THE PREVENTION AND CONTROL OF PERTUSSIS

MODIFICATION OF THE IMMUNE RESPONSE
BY PERTUSSIS VACCINE

Chairman: *M. S. Zakharova*
Rapporteur: *A. H. Griffith*

Introductory Statement

M. S. Zakharova

It has been suggested that the prospects for improved vaccines and methods for the prevention and control of pertussis be discussed during this final session. It is necessary to consider again some available information on antigens and other immunobiologically active substances as well as the metabolism and genetics of *B. pertussis.*

Discussions during previous sessions make it evident that all available ways of improving whole cell pertussis vaccine have not been exploited, but constraints on time do not permit a comprehensive analysis of all factors concerned with efficacy and reactogenicity of pertussis vaccine.

I think most of our discussions should have been concerned with new purified vaccines and methods to prepare them, with prospects for their use in children, and with the main considerations involved in the development of noncellular *B. pertussis* vaccines. Despite numerous attempts to obtain purified antigens of high potency for immunization against whooping cough, we still do not have a preparation more effective and less reactogenic than pertussis whole cell vaccine. Four or five purified preparations have already been used for immunization of a limited number of children with promising results, but they have not yet been subjected to the rigors of large-scale field trials.

The need to develop a simple method for obtaining a preparation relatively free from unwanted biologically active substances has become particularly important in view of the establishment of the WHO Expanded Program on Immunization in 1976.

Some progress has been made during the last decade in the elucidation of certain molecular properties and the location in the cell of protective antigen, hemagglutinin, agglutinogens, HSF, LPF, the adjuvant substances, and the relationships that exist between these components. Though the data are not comprehensive, they raise the possibility that cellular entities responsible for protection against infection can be separated from undesirable entities. Accordingly, we should develop laboratory control testing for such vaccines as well as for the whole cell vaccine.

The establishment of a new international standard for pertussis vaccine remains a problem. The present standard was prepared 25 years ago. In my opinion it can be used to compare vaccines from different manufacturers, but we probably cannot use it any longer to determine the amount of material (cells or purified antigens) in a human immunizing dose of present vaccines and particularly of the new preparation. The problem of correlating laboratory potency assays with the efficacy of pertussis and DTP vaccines in the field may be discussed during this session, since it has not been discussed during previous sessions.

Let us now hear from the speakers who will present their work on various aspects of the problems involved in the development of new vaccines. Also to be presented during this session will be studies on the modification of the immune response by pertussis vaccine.

Theoretical Outlines on the Preparation of a Noncellular Pertussis Vaccine

M. S. Zakharova

ABSTRACT

The development of noncellular pertussis vaccines depends on information concerning the molecular parameters, localization, and relationship of numerous immunobiologically active substances of the *Bordetella pertussis* cell. From experimental data one may conclude that of all the antigenic components of the organism only the protective antigen (a structure of the cytoplasmic membrane called protectosome) and hemagglutinin (fimbriae) are concerned with protection against infection. Both are proteins.

Toxic substances, such as the heat labile and heat stable toxins, the lymphocytosis promoting factor (LPF), and the histamine sensitizing factor (HSF) are components of the cell wall and cytoplasm. They do not directly relate to protection (loss of the adjuvant effect in combined vaccines can be compensated by the addition of mineral or other adjuvants), but it is evident that they may participate in the production of untoward reactions to vaccination.

The cell wall contains most agglutinogens, which are low molecular weight proteins determining the serological specificity and are not related to HSF, LPF, and hemagglutinin. They have no protective properties, but they do have sensitizing properties. When preparing protective antigens one should strive for maximal retention of the cytoplasmic membrane and inner layers of the cell wall as well as removal or inactivation of its outer structures.

Vaccines of this type can be obtained by 1) isolating cellular components responsible for protection while removing or inactivating all other biologically active substances as much as possible, 2) regulating the synthesis of the protective and toxic proteins during cultivation of organisms, and 3) obtaining genetic mutants lacking toxicity or other harmful properties.

Despite numerous attempts to isolate purified antigens of high potency for immunization against whooping cough, medical science has failed to produce a preparation more effective and less reactogenic than pertussis whole cell vaccine. The development of a simple method of isolating an antigenic preparation devoid of unwanted biological activity, including that responsible for postvaccination side effects in children, remains an important problem, particularly in relation to the Expanded Program on Immunization initiated by the World Health Organization in 1976.

Review articles by Pittman (1), Munoz (2), Morse (3), the monograph by Munoz and Bergman (4), other recent publications (5–11), and our own investigations have led us to certain conclusions, which we believe will contribute to the solution of this problem.

Bordetella pertussis has a complex morphological structure that includes many antigenic and other biologically active substances. On injection into humans or animals, these substances induce diverse immunological, toxicological, and other reactions. Evidently not all antigenic substances of the cell participate equally in the formation of immunity against infection.

Unlike many other Gram negative pathogens, two activities of *B. pertussis* protectogenicity, a better term than "protection," and agglutinogenicity are determined by cellular substances. This fact has been responsible for many futile attempts to isolate a "complete antigen." The lipopolysaccharides (LPS) of *B. pertussis* induce bactericidal antibodies but they are not involved in protection, and bactericidal antibodies are not the same as those produced in response to mouse protective antigens (2).

More fruitful lines of investigations were followed during the last decade. They included the study of the main properties of immunobiologically active substances of *B. pertussis* at subcellular and molecular levels, their relationship to certain morphological structures, and their location in the cell. As a result, important information was obtained

on the molecular parameters and cellular location of the following activities: protective (PA), agglutinogenic (AgA), hemagglutinating (HA), lymphocytosis promoting (LPA), histamine sensitizing (HSA), heat labile toxin (HLT), and others. Some of the questions concerning the structural and functional interrelationships between these activities have been elucidated.

Figure 1 shows schematically the location of the main immunobiologically active substances of *B. pertussis*. Table 1 contains data we believe are important for theoretical substantiation of a rational method for obtaining an effective vaccine free of the toxic and other harmful substances in the microbial cells.

Of all known antigenic components of *B. pertussis*, only protective antigen and hemagglutinin create protection against infection. The protective activity of *B. pertussis*, as shown by Sato and his coworkers (12,13) and in our investigations (14,15), is connected with spiral-shaped structures we call protectosomes. They consist of lipoprotein (76–79% protein and 10–23% lipid), are located on the plasma membrane, and penetrate into the inner layer of the cell wall. When the cell wall is detached protectosomes remain on the plasma membrane. The sedimentation constant of these molecules is 22S and the molecular mass is 860,000 ± 40,000 daltons (Fig. 2). Protectosomes do not possess HSA and LPA. The spiral thread of the protectosome is 9 nm long and consists of several subunits. The protective activity is determined by the protein part of the molecule, while toxicity is determined by the lipid. Protectosomes lose their protective activity when treated with 6M urea at alkaline pH and dissociate into subunits (sedimentation constant: 3S; molecular mass: 40,000 daltons). The purified preparation of the protectosomes has no HLT, LPA, or HSA, and only small amounts of AgA. Protectosomes isolated from *B. pertussis* strains of different serotypes have equal protective activity.
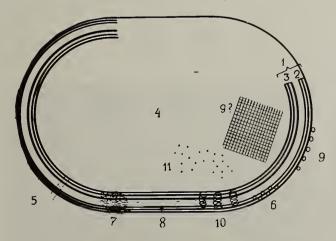
Thus it has been established for the first time that protective activity is related to certain definite morphological structures of cells.

These data not only radically change the commonly held view on the primary importance of surface structures of the cell (capsule, wall) in the development of immunity against infection but also question the validity of searches for so-called soluble antigens.

The preparation of vaccine (ether pertussis antigen) from cell fragments of *B. pertussis* brings practical confirmation of the protective role of protectosomes (16). This preparation was obtained by ultrasonication of the cell suspension followed by ether treatment (1:2.5). After the ether layer was removed and merthiolate (1:10000) added, the preparation contained about 44 to 64% of the protective activity (IPU) of the original suspension. Adsorption of the ether extract pertussis antigen (EPA) on $AlPO_4$ (AEPA) increased its activity by 1.5 to 3.3 times (Table 2) with yields of 70 to 142%.

Electron microscopy of EPA sedimented at 105,000 × g revealed membranes with residual cytoplasm and protectosomes (Figs. 3 and 4). The toxicity and sensitizing properties of the preparations were considerably reduced (17).

Three small groups of infants were immunized with these vaccines. The percentage of systematic reactions after immunization of children with EPA and AEPA was 4–5 times lower than after vaccination with pertussis vaccine.

By orderly disintegration of cells followed by treatment with salt solutions of different ionic strength and with lysozyme, we obtained (7,18) materials free of cell wall, such as mureinoplasts, which retained peptidoglycan, and protoplasts. The plasma membrane fraction was isolated from the ultrasonic disintegrate of protoplasts. The fractions of mureinoplasts, protoplasts, and plasma membrane (Figs. 5–7) were studied in various tests to determine their protective, toxicological, and sensitizing properties. Protective activity was retained by all three fractions, but it was less than in the un-

Figure 1. *Bordetella pertussis* (location of immunobiological activities in cell). 1) envelope; 2) cell wall; 3) plasma membrane; 4) cytoplasm; 5) pili (HA + PA); 6) protein globules (HSF + HA + LPF); 7) agglutinogens; 8) HST (endotoxin); 9) HLT; 10) protectosomes; 11) ribosomes.

Table 1. Characteristics of Immunobiological Activities of *Bordetella pertussis* (purified state)

Characteristic	Protective Antigen (PA), Protectosome	Hemagglutinin (HA)			Major Agglutinogens			Leucocytosis-Promoting Factor (LPF)	Histamine-Sensitizing Factor (HSF)	Heat Labile Toxin (HLT)	Heat Stable Toxin (HST), Endotoxin (O-antigen)
		HA	HA I	HA II	1	2	3				Lipopolysaccharide (LPS)
Chemical composition: 1) Protein 2) Carbohydrate 3) Lipid (2, 6, 8, 13, 14, 26, 27, 43, 47) [a]	76% 2% 23% Specific determinant is a protein.		83% 6.8% 4.7%	47.7% 25.0% 23.9%	+ / −	94% 2.5% 3.5%	78% 4.6% 2.5%	70-80% 1% −	30.6 2.1 (reducing sugars) 5)	+ / −	‡
Constants, sedimentation (6, 13, 26, 27, 29, 43, 46)	22S		6.3S	5.3S		0.47S	0.82S		1S		42-45S
Molecular weight (kilodaltons) (6, 8, 13, 26, 29, 47)	860±40		~133	~103		10-20 Both consist of one polypeptide chain. N amino acid residues: 97	23 71	~87 Consists of four polypeptide chains.	86-94		Approx. 10⁶
Appearance in electron microscopy, size (8, 9, 13, 14, 20, 43)	1) Cylindrical molecules 12x12 nm composed of four layers of ring-shaped substructures consisting of several particles arranged in a petal-like pattern (13) 2) Spiral structure. In horizontal plane, hexahedron 8-12 nm in diameter, vertical dimension 11-12 nm. Consisted of four 1.2-1.5 nm separate discs (14)	Filaments 2.0-2.5 nm x 60-100 nm or 2x40 nm	Filaments 2x40 nm	Spherical particles 6 nm in diameter	Not known[b]			1) Filamentous molecules ~2x40 nm and spherical ones of ~6 nm 2) Ring-shaped particles 7.5-8.0 nm in diameter. Each ring composed of 4-5 subunits 2.8 nm in diameter	The same round particles in which LPF is found (two determinants)		Rod-shaped molecules of various lengths, 5.0-10.0 nm in diameter
Location (8,9,10,13,14)	Plasma membrane, inner layer cell wall	Pili	Fimbriae (pili)	Cell wall	Envelope and especially in cell wall			Cell wall, fluid, and solid media after *B. pertussis* growth		Periplasmic Supernatant of fluid culture *B. pertussis*	Cell wall
Relation to other antigens (activities) 8,9,20,25,28,46	Agglutinogen. Toxic substances	None	None or PA	HSF, LPF	None	None	None	HSF	LPF or freedom from it Strong affinity to foreign proteins	None	None
Role in prophylaxis of whooping cough (2,4,8,9,13,14, 20,25,32,43)	Main protective antigen (PA)	None or unknown	PA	None	None	None	None	None	None	None	None

[a] References
[b] No data

Figure 2. Electron micrograph: a) The 22S antigen, negatively stained with 1% phosphotungstic acid, b) a trypsin-treated cell suspended in 0.05 M phosphate buffer, pH 8.0 incubated with 0.1% trypsin at 37° for 20 min., negatively stained with 1% phosphotungstic acid. Reference 13.

Table 2. Protective Activity of EPA and AEPA

Lot	Number of IPU/ml (geometric mean)	
	Without adsorbent	After addition AlPO$_4$
1(2)	5.09	16.14
2(3)	7.62	25.2
3(4)	12.88	19.5

Figure 3. Morphology of ether pertussis antigen (EPA), ultra-thin section of preparation (120,000×).

Figure 4. Fraction of protectosome isolated by ultracentrifugation from EPA (105,000 × g), negatively stained with 1% phosphotungstic acid (200,000×). Reference 16.

treated microbial suspension and greater in protoplasts than in plasma membrane. The reason for this is not clear. The protective activity of the plasma membranes was 25–35% of that in the parent microbial suspension. Addition of aluminum phosphate increased the protective activity of all fractions. This occurred also with EPA preparations, but it was most pronounced in plasma membrane preparations (sixfold rise).

These cell disintegration studies showed that toxic substances (HSA and LPA) could be removed or inactivated and proved once again that they were located mainly in the cell wall. We failed to detect toxic properties in plasma membrane preparations by the methods available (Fig. 8). Other experiments established that HLT is located not only in the cytoplasm, but also in the periplasm (10,19).

The location of protectosomes in the plasma membrane provides evidence that this cell structure is more important in host protection than are surface structures of the cell wall, which contain toxic and sensitizing substances.

Hemagglutinating activity of *B. pertussis* has been identified with pili (6,8,9,20). According to recent studies by Sato (6,9) purified HA, a protein with a molecular weight of approximately 133,000,

Table 3. Protective Properties

Preparation	IPU/ml	
	Without adsorbent	With AlPO$_4$ (mg/ml)
Vaccine	40.3	none
Mureinoplast	11.7	34.0
Protoplast	13.5	30.2
Plasma membrane	7.4	52.0
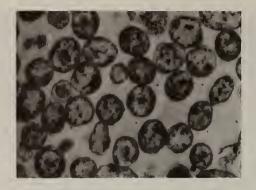
Figure 5. Protoplasts of *B. pertussis*, strain gL 353, ultrathin section (66,000×).

has been isolated from pili (filaments 2 × 40 nm) and differs from the other HA, a globular protein associated with LPA, which lacks protective activity. Nakase (20) also isolated a highly purified HA by physical and chemical methods. It had a hemagglutinating titer of 100,000 and had no protective activity, HSA or LPA, but morphologically it consisted of 2 × 40 nm filaments composed of protein with carbohydrates and lipid. The HA was inactivated by heating at 56° for 50 minutes. The cause of this discrepancy may be elucidated by further investigations. Meanwhile the use of this unstable component of the cell for the preparation of vaccine does not seem to be sufficiently substantiated.

In recent years the role of agglutinogens in the production of immunity against pertussis and in type specificity of protection has attracted much attention (21,22,23,24). It is known that isolated purified agglutinogens 1, 2 and 3, which are low molecular weight proteins, located mainly in the cell wall, and their antisera afford no active or passive protection to mice against intracerebral challenge by *B. pertussis* (25–33).

Figure 6. Protoplasts of *B. pertussis*, strain gL 353, ultrathin section (160,000×).
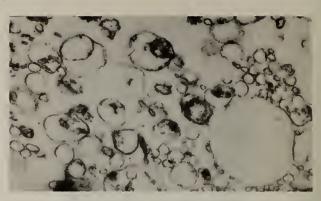
Figure 7. Plasma membrane *B. pertussis*, strain gL 353, ultrathin section (18,000×). Reference 18.

Several reports show that the composition of agglutinogens of *B. pertussis* strains is independent of their protective antigens. Thus the loss of agglutinogens during spheroplast formation (5,35), variations in culture media, or treatment with mutagens (strain gL 353) is not accompanied by a decrease in the protective properties of cells. Conversely, the protective properties decline sharply on prolonged storage, but their agglutination ability remains unchanged (36). The removal of agglutinins from
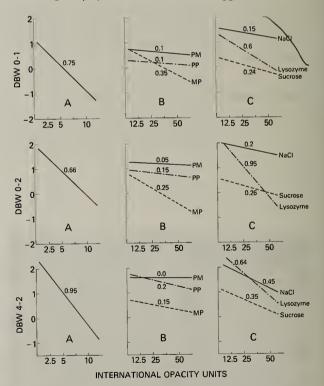
Figure 8. Toxicity of different fractions of *B. pertussis*, strain gL 353 (weight gain test). Regression lines: A. original vaccine; B. fraction of mureinoplasts (mp), protoplasts (pp), and plasma membrane (pm); C. the solution after washing of the cells (0.5 M NaCl, sucrose and lysozyme).

a bacterial agglutinating serum by adsorption does not change its protective properties (5,37). An EPA preparation with high protective activity appeared to be a weak agglutinogen (17). Finally, the immunization of children with Pillemer's antigen (SPA) revealed good protective properties against infection but a weak agglutinin response (38).

The shift in prevalence of serotype of strains isolated from pertussis cases is independent of agglutinogenic composition of vaccines used in immunization programs and is not necessarily followed by changes in morbidity (39–41).

Whatever the final decision will be, it is becoming increasingly evident that agglutinogens are not required for protection of mice against infection. All available information leads to the conclusion that agglutinogens play little or no role in protection against pertussis in children.

All other biologically active substances and their potential capacity to induce side reactions after vaccination have been described and discussed (1,2,4). We will not review them here, but stress again that it is reasonable to remove from preparations those substances which contain HLT, HST, LPA, and HSA. The removal of the lipopolysaccharide, which is probably the main adjuvant of the cell, may be compensated by replacement with mineral or other adjuvants.

In conclusion, it appears that various approaches are available for the development of an effective vaccine with maximally decreased reactogenic properties. They are: 1) isolation of cell components responsible for protection, such as plasma membrane, protectosomes, and HA—devoid of all other unwanted biologically active substances including agglutinogen 2, which has sensitizing properties; 2) regulating the synthesis of protective and toxic proteins during cultivation of microbe cells; and 3) obtaining genetic mutants devoid of toxicity or other unwanted properties.

It is probable that the established relationship between protectosomes and glutamine synthetase (42) opens a prospect for regulation of this important specific protein synthesis, since the determination of this enzyme activity may prove to be a useful index of the intensity of protectosome synthesis in bacterial cells.

REFERENCES

1. Pittman, M. *Bordetella pertussis*—bacterial and host factors in the pathogenesis and prevention of whooping cough. Infectious agents and host reactions (Mudd S., ed). Philadelphia: W. B. Saunders Co.; 1970:239–270.

2. Munoz, J. Protein toxins from *Bordetella pertussis*. Microbial Toxins, vol. II. New York: Academic Press, Inc.; 1971:271–300.

3. Morse, S. I. Biologically active components and properties of *Bordetella pertussis*. Adv. Appl. Microbiol. 20:9–26; 1976.

4. Munoz, J.; Bergman, R. *Bordetella pertussis*, Immunology series, Rose, N. ed., Marcel Dekker, Inc., New York and Basel, v. 4, 1977.

5. Dolby, J. M.; Bronne-Shanburg, C. The use of spheroplast-derived strains to differentiate between *B. pertussis* heat labile agglutinogens and protective antigen for mice. J. Biol. Stand., 3(I):89–100; 1975.

6. Arai, H.; Sato, Y. Separation and characterization of two distinct hemagglutinins contained in purified leucocytosis-promoting factor from *Bordetella pertussis*. Biochim. et Biophys. Acta 444:765–782; 1976.

7. Zakharova, M. S.; Gureeva, A. A.; Pereversev, N. A. Isolation of the individual structural elements of the gram-negative bacteria of the *Bordetella* genus and study of their properties. Report I. Formation of mureinoplasts, true protoplasts and other microbial variants from *B. pertussis*. Zh. Mikrobiol. Epidemiol. Immunobiol., 8:45–48; 1975.

8. Morse, S. I.; Morse, J. H. Isolation and properties of the leucocytosis and lymphocytosis-promoting factor of *Bordetella pertussis*. J. Exp. Med. 143:1438–1502; 1976.

9. Sato, Y. Biological significance of *B. pertussis* fimbriae or hemagglutinin. Infection and immunity in whooping cough. Abstracts of All-Union symposium on immunology and epidemiology edited by M. Zakharova et al., Minsk, 64–65; 1977.

10. Lapaeva, I.; Soderholm, J.; Mebel, S.; Pereversev, N. Concerning the localization and properties of some immunologically active substances of *B. pertussis* (Intact and with a defective cell wall). Actual questions of epidemiology and immunology of whooping cough, eds. O. Baroyn and M. Zakharova et al., Moscow, 1977, 63–68.

11. Ackers, J.; Dolby, J. M. The antigen of *Bordetella pertussis* that induces bactericidal antibody and its relationship to protection of mice. J. Gen. Microbiol. 70:371–382; 1972.

12. Sato, Y.; Nagase, K. Isolation of protective antigen from *Bordetella pertussis*. Biochem. Biophys. Res. Commun. 27:195–201; 1967.

13. Sato, Y. Isolation and some properties of protective antigen of *Bordetella pertussis*, Symp. Series Immunobiol. Standard., Internat. symposium on pertussis vaccine, 13:214–220, Karger, Basel, 1970.

14. Zakharova, M.; Lapaeva, I; Pereversev, N. Separation of biologically active components from *B. pertussis*. Symp. Series Immunobiol. Standard., Internat. symposium on pertussis vaccine, 13, 227–233, Karger, Basel, 1970.

15. Lapaeva, I. A.; Zakharova, M. S.; Pereverzev, N. A. Isolation and certain properties of a protective *B. pertussis* antigen. Zh. Mikrobiol. Epidemiol. Immunobiol. 11:27–30; 1970.

16. Zakharova, M. S.; Shirko, G. N.; Gureeva, A. A.; Pereverzev, N. A. Vaccine from the cell fragments of *B. pertussis*. Report I. Protective, sensitizing properties and morphological characteristics of the vaccine. Zh. Mikrobiol. Epidemiol. Immunobiol. 11:103–108; 1976.

17. Shirko, G. N.; Zakharova, M. S. Vaccine from cellular *B. pertussis* fragments. Report II. Toxic properties of the vaccine. Zh. Mikrobiol. Epidemiol. Immunobiol. 7:68–74; 1978.

18. Gureeva, A. A.; Pereverzev, N. A. Isolation of some structural elements of gram negative bacteria of the genus *Bordetella* and a study of their properties. Report II. Isolation of the fraction of cytoplasmic membranes from the protoplasts *B. pertussis* and a study of their chemical and some enzymatic properties. Zh. Mikrobiol. Epidemiol. Immunobiol. 10:88–94; 1976.

19. Gureeva, A. A.; Zakharova, M. S. On the question of the localization of some toxic substances of the pertussis microbe. Actual question of epidemiology and immunology of whooping cough, eds. O. Barojan and M. Zakharova, Moscow, 69–72, 1977.

20. Nakase, J.; Doi, M.; Kasuga, T. Hemagglutinating substance of *B. pertussis*. 21st Annual Meeting. International symposium on bacterial toxins, 8–14; 1974.

21. Preston, N. W. Type-specific immunity against whooping cough. Br. Med. J. 2:724–726; 1963.

22. Preston, N. W. The epidemiological situation in various countries. Serotypes. International symposium on pertussis, Bilthoven, Symp. Series Immunobiol. Standard. 13: 18–20, Karger, Basel, Munich, New York, 1970.

23. Preston, N. W. Potency tests for pertussis vaccine: doubtful value of intracerebral challenge test in mice. J. Path. and Bact., 91(1):173–179; 1966.

24. Preston, N. W.; Stanbridge, T. N. Mouse or man? Which are pertussis vaccines to protect? J. Hyg. (Camb.) 76:249; 1976.

25. Zakharova, M. S.; Denisova, T. P. Identification of an antigen isolated from *B. pertussis* with properties of factor 2 of agglutinogen. Zh. Mikrobiol. Epidemiol. Immunol. 6:92–94; 1969.

26. Zulina, L. V.; Denisova, T. P.; Korgienko, V. P. Chemical composition and physicochemical properties of agglutinogen of *B. pertussis* (factor 2). Zh. Mikrobiol. Epidemiol. Immunol. 9:195; 1972.

27. Zulina, L. V.; Zakharova, M. S.; Bagdasarova, I. P. Obtaining and chemical characteristics of agglutinogen with the properties of factor 3. Zh. Mikrobiol. Epidemiol. Immunol. 3:101–104; 1972.

28. Zakharova, M. S.; Zulina, L. V.; Bagdasarova, I. P. Role played by agglutinogen 3 in the immunity against *B. pertussis*. Zh. Mikrobiol. Epidemiol. Immunol. 6:78–80; 1972.

29. Amelina, I. P. Physical-chemical and serological study of agglutinogen 3 of *B. pertussis*. Zh. Mikrobiol. Epidemiol. Immunol. 2:64–66; 1976.

30. Eldering, G.; Holwerda, J.; Baker, J. *Bordetella pertussis* culture having only species factor 1. J. Bacteriol. 91(5): 1759–1762; 1966.

31. Eldering, G.; Holwerda, J.; Baker, J. Mouse-protective properties of *Bordetella pertussis* serotypes in passive tests. J. Bacteriol. 93(6):1758–1761; 1967.

32. Ross, R.; Munoz, J. Antigens of *B. pertussis*, V. Separation of agglutinogen and mouse protective antigen. Infect. Immun. 3(2):243–248; 1971.

33. Nakase, J.; Kasuga, T. Studies on the constituents of *Bordetella pertussis*. Kitasato Arch. Exp. Med. 35: 1–4; 1962.

34. Holt, L. B.; Spasojevic, V. The role of surface antigens in the protective potency of *Bordetella pertussis* suspensions as measured by the intracerebral challenge technique in mice. J. Med. Microbiol. 1:119–126; 1968.

35. Lapaeva, I. A.; Shirko, G. N.; Zakharova, M. S. Characteristics of serological variants of *Bordetella pertussis* with a defective cell wall. Zh. Mikrobiol. Epidemiol. Immunol. 4:23–27; 1974.

36. Zakharova, M. S. The significance of the agglutinogens in immunity against whooping cough. The droplet children infections. Health, Kiev, 52–54; 1971.

37. Hink, J. H.; Johnson, F. F. Preparation of antigen mixture from *H. pertussis* by selective denaturation. J. Immunol. 57:323–330; 1947.

38. Medical Research Council. Vaccination against whooping cough. Br. Med. J. 1, 994–1000; 1959.

39. Zakharova, M. S. Immuno-chemical basis of serological specificity and serotypes of *B pertussis*. III National Confer. on bacterial infections and immunology, Sofia, 1, 229–236; 1974.

40. Zakharova, M. S.; Vorobjeva, A. I.; Gorbunova, Z. V. Etiological structure of whooping cough in Estonia SSR. Zh. Mikrobiol. Epidemiol. Immunol. 1:136; 1973.

41. Raška, K. Whooping cough—Epidemiological situation in the world. International symposium on pertussis vaccine. 13, 4–9; Karger, Basel, 1970.

42. Zakharova, M. S.; Julina-Zlateva, L. V.; Lapaeva, I. A. Glutamine synthetase of *Bordetella pertussis*. Whooping cough, Abstracts of All-Union symposium on immunology and epidemiology, eds. M. Zakharova et al., Minsk, 64–65, 1977.

43. Nakase, Y.; Tateisi, M.; Sekiya, K.; Kasuga, T. Chemical and biological properties of the purified O-antigen of *Bordetella pertussis*. Jpn. J. Microbiol. 14(1):1–8; 1970.

44. Kasai, N. Chemical studies on the lipid component of endotoxin, with special emphasis on its relation to biological activities. Ann. N. Y. Acad. Sci. 133:486–507; 1966.

45. Aprile, M. A.; Wardlaw, A. C. Availability and specificity of lipopolysaccharide on the surface of 56° C heated *Bordetella pertussis*. Can. J. Microbiol. 19:536–538; 1973.

46. Homma, R.; Shimazaki, Y.; Funasaka, I.; Kuratsuka, K. Outline of purification and some properties of histamine-sensitizing factor from *Bordetella pertussis*. Jpn. J. Med. Sci. Biol. 22(2):123–126; 1969.

47. Lehrer, S.; Tan, E.; Vaughan, J. Extraction and partial purification of the histamine-sensitizing factor of *Bordetella pertussis*. J. Immunol. 113(1):18–26; 1974.

Extraction and Fractionation of *B. pertussis* Antigens In Order To Reduce The Reactogenic Properties of Pertussis Vaccines

J. Nagel and S. de Graaf

ABSTRACT

Mouse protective antigen (MPA) was precipitated wth 25% polyethylene glycol (PEG) from ultrasonic and sheared extracts of *B. pertussis* cells (strain 509). In polyacrylamide gel electrophoresis in the presence of SDS, an MPA and a leukocytosis promoting factor (LPF) band could be recognized. If the molecular weight of PEG did not exceed 1,000, precipitates with low LPF activity were obtained from sheared extracts. The precipitates obtained with PEG 300 yielded about 30% of the MPA and about 10% of the endotoxin originally present in the extract.

These results could be confirmed in a mouse weight-gain test and in a sensitization test in which mice received a second injection with pertussis cells. The optimal conditions of the PEG fractionation of sheared extracts and the effect of the reduction of the LPF and endotoxin content of pertussis vaccines on the incidence of major vaccination reactions remain to be studied.

There have been many publications on the incidence of major neurological reactions occurring after immunization of infants with pertussis vaccines. The component or components of the pertussis cells that may be responsible for these reactions are still unidentified.

In animal models four toxic activities can be demonstrated. They are the heat labile toxin, which is readily destroyed by heating or by formalin treatment; the heat stable endotoxin; the component that causes a hypersensitivity to histamine and various other agents and provokes a leukocytosis (HSF/LPF); and the islet-activating protein (IAP), which potentiates the insulin production (1).

Endotoxin, so-called HSF or LPF, and IAP deserve special attention in relation to vaccination reactions. The toxicity of endotoxin in animals and man is well known, and according to Griffith (2) some adverse vaccination reactions may be caused by its presence in pertussis vaccines. On the other hand, these reactions might also be due to the presence of HSF/LPF, although the relationship between sensitization in animals and reactions in children is not clear. Finally, IAP profoundly influences carbohydrate metabolism in animals. In pertussis-vaccinated animals an increased plasma insulin level can occur and will reduce the hyperglycemic response to epinephrine (3,4). This also occurs in children after pertussis vaccination (5,6).

The aim of our studies was to separate mouse protective antigen (MPA) from these unwanted activities.

This paper describes the extraction of *B. pertussis* antigens by mechanical means and the fractionation of these extracts by PEG.

We chose mechanical disintegration of pertussis cells for two reasons. First, it was found in the British field trials that Pillemer's vaccine (7) obtained by ultrasonic treatment of strain 134 bacteria protected children very well, although it proved too toxic for routine immunization. Second, chemical extraction resulted in a poor recovery of the MPA.

The various preparations obtained were analyzed in the limulus lysate reaction for the determination of the endotoxin, in mouse protection and LPF tests, and by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) as described by Laemlli (8).

Comparison of the results of disc gel electrophoresis with the biological activities of the preparations gave us the opportunity to attribute two lines to MPA and LPF, respectively. Although we have no final proof for this assumption, none of our results conflicts with it. In the fractionation studies these lines appear to be good indicators of those biological activities. Some of the patterns we obtained are shown in Figure 1. On the left are the
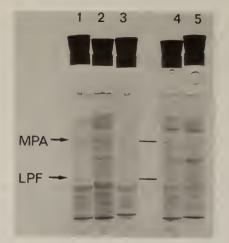
Figure 1. SDS–PAGE: patterns of ultrasonic (1) and shearing (3) extracts of cells, strain 509 (2); cells, strain 134, grown in media with low (4) and high (5) Mg++ concentration.

MPA →
LPF →

strain 509, cultivated in a modified Cohen-Wheeler medium. The bacterial cells were harvested by acid-precipitation (pH 3.5–4) and resuspended in phosphate buffer, pH 7.5, containing 0.5 M NaCl.

The cells were distintegrated by ultrasonication or by shearing. For ultrasonication a Lab-Sonic apparatus was used, applying a power of 40 W at 200 kHz for 30 minutes. Shearing was done with a distintegrator as described by Novotny (10) using glass beads with a diameter of 0.1 mm, stirring them with a concentrated cell suspension at a speed of about 2,000 rpm for 10 minutes. After shearing, the glass beads were rinsed with the phosphate buffer.

After disintegration, the suspensions were treated with DNase and incubated for 15 minutes at room temperature to reduce the viscosity. They were then centrifuged and the supernatant fluids collected. The results showed that with both methods of distintegration at least 75% of the antigens (MPA, HSF, LPF, and ET) were recovered in the supernatants.

Fractionation of these extracts was done by PEG precipitation, using a final concentration of 25% PEG. We concluded that the results of the fractionation were strongly dependent on the molecular weight (MW) of the PEG used (Fig. 2). PEG with a MW of 600 or 1,000 precipitated about 50% of

patterns of a cell suspension (strain 509) and two extracts derived from it, and on the right are those of two cell suspensions (strain 134) cultivated in media containing normal and increased Mg++ concentration respectively. In accordance with the results obtained by Parton and Wardlaw (9), the latter suspension showed a low MP activity, which is confirmed by the absence of the MPA band in the SDS–PAGE pattern.

In our fractionation studies, we used B. pertussis

Figure 2. Estimated recovery of MPA, LPF, and endotoxin from shearing and ultrasonic extracts by precipitation with 25% of PEG of different molecular weight (mw).

the MPA. At lower MW, probably less MPA was precipitated.

Very low LPF activities were found in the 25% PEG 600 and PEG 1,000 of the sheared extracts, but not with the ultrasonic extracts. For this reason, sheared extracts are preferable to ultrasonic extracts.

Figure 2 shows that the endotoxin and the MPA behave almost identically, supporting the hypothesis that the MPA may be part of the L-layer of the cell wall (11). Ways will have to be found to reduce the toxicity of endotoxin if it cannot be removed without losing the MPA.

The fractions obtained by precipitation with 25% PEG 600 and 1,000 were also examined in SDS–PAGE (Fig. 3). The LPF-band is found only in the supernatant fractions, whereas the MPA is almost equally divided between the supernatant and the precipitate.

The toxic properties of acid-precipitated cells, a sheared extract prepared from them, and a 25% PEG 1,000 precipitate were compared in a mouse weight-gain text. Groups of ten 14–17 g mice were injected with 40 International Opacity Units (IOU). The weight change curves for the three preparations are shown in Figure 4. From the mean weight change after 1 day it is clear that there is very little difference in the endotoxin content of these preparations. However, the differences in mean body-

Figure 3. SDS–PAGE: Results of precipitation with 25% PEG: ultrasonic extract (1), PEG 600-precipitate (2), supernatant (3); PEG 1000-precipitate (4), supernatant (5).

weight change after 7 days, reflecting differences in LPF content, are pronounced.

The toxicity test was extended by giving all mice a second injection with a dose of 10 IOU of acid-precipitated cells 1 week after the first injection (Table 1). The mortality in each group was determined 1 week later. All mice survived in the group initially injected with PEG 1,000 precipitate, but a mortality rate of about 40% was found in both of the other groups. We concluded that the PEG precipitate did not show any sensitizing activity. This again confirms the absence of the HSF/LPF complex.
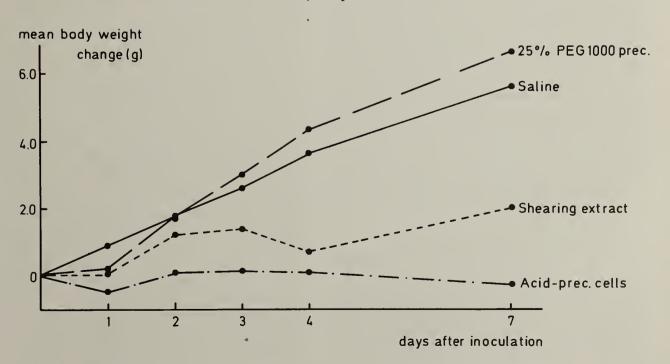
Figure 4. Mouse weight-gain test (dose~40 IOU).

Table 1. Mortality of Mice Initially Injected With Various *B. pertussis* Preparations[a] One Week after a Second Injection with Pertussis Cells (10 IOU)

	Mortality (%)
Acid precipitated cells	50
Sheared extract	30
PEG 1000 precipitate	0
Control	0

[a] dose corresponding to 40 IOU

Further investigations are in progress to determine the IAP content of these precipitates and to assess its importance in pertussis vaccines.

REFERENCES

1. Yajima, M.; Hosoda, K.; Kanbayashi, Y.; Nakamura, T.; Nogimori, K.; Mizushima, Y.; Nakase, Y.; Ui, M. Islets-activating protein (IAP) in *Bordetella pertussis* that potentiates insulin secretory responses of rats. Purification and characterization. J. Biochem. (Tokyo) 83:295–303; 1978.

2. Griffith, A. H. Reactions after pertussis vaccine: a manufacturer's experiences and difficulties since 1964. Br. Med. J. 1:809–815; 1978.

3. Tabachnick, I. I. A.; Gulbenkian, A. Adrenergic changes due to pertussis: insulin, glucose and free fatty acids. Eur. J. Pharmacol. 7(2):186–195; 1969.

4. Yajima, M.; Hosoda, K.; Kanbayashi, Y.; Nakamura, T.; Takahashi, I.; Ui, M. Biological properties of islets-activating protein (IAP) purified from the culture medium of *Bordetella pertussis*. J. Biochem. (Tokyo) 83:305–312; 1978.

5. Hannik, C. A.; Cohen, H. Changes in plasma-insulin concentration and temperature of infants after pertussis vaccination. This conference.

6. Sen, D. K.; Arora, S.; Gupta, S.; Sanyal, R. K. Studies of adrenergic mechanisms in relation to histamine sensitivity in children immunized with *Bordetella pertussis* vaccine. J. Allergy Clin. Immunol. 54:25–31; 1974.

7. Pillemer, L.; Blum, L.; Lepow, I. H. Protective antigen of *Haemophilus pertussis*. Lancet I: 1257–1260; 1954.

8. Laemlli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685; 1970.

9. Parton, R.; Wardlaw, A. C. Cell envelope proteins of *Bordetella pertussis*. J. Med. Microbiol. 8:47–57; 1975.

10. Novotny, P. A simple rotary disintegrator for microorganisms and animal tissues. Nature 202:364–366; 1964.

11. Novotny, P.; Cownley, K. Free and phenol endotoxin of *B. pertussis*. Soc. Gen. Microbiol., Proceedings 4:10; 1976.

An Effective Procedure for Preparing Extracted Protected Antigen from *Bordetella pertussis*

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ABSTRACT

Bordetella pertussis microorganisms were treated at 4° C with a variety of extracting solvents followed by high speed centrifugation to remove particulate matter. Protein analysis of the ultracentrifuge supernatants showed highly variable yields depending on the method of extraction used. Simple salt extraction solubilized only marginal quantities of protein antigens, whereas more aggressive agents such as urea or anionic detergents increased the yield five- to tenfold. These data were corroborated by SDS gel electrophoretic analysis of the extracts. Several components absent in salt extracts were prominent in preparations derived from cells treated with detergents. Concomitantly, the yield of protective activity as measured by the mouse potency assay was considerably elevated in these extracts. Electrophoretic analysis may be a suitable way to monitor fractionation of the many protein components solubilized by detergent treatment.

A reasonable yield of cell-free protective antigen complex was obtained by a procedure involving the treatment of *Bordetella pertussis* cells with 6 M urea, 1 M NaCl in 0.1 Tris-HCl buffer, pH 8.0, followed by removal of particulate matter by centrifugation. The solubilized antigens were adsorbed on a mineral carrier before detergent was withdrawn. This minimized the formation of aggregates and subsequent loss of antigenic material. This adsorbed cell-free preparation may be an alternative to the conventional whole-cell material for active immunization against *Bordetella pertussis* infection.

INTRODUCTION

During the last 30 years, solid evidence has accumulated that the protective antigen complex of *Bordetella pertussis,* defined by its ability to induce protective immunity in mice against intracerebral challenge with virulent homologous organisms, is localized on the surface of these bacteria (1–3). Although numerous strategies have been devised in attempts to solubilize the relevant components from the outer membrane region of this pathogen, perhaps the most frequent approach has involved the use of salt solutions, often in combination with sonication, followed by centrifugation to remove the bacterial cells (4–6). However, large scale preparation of the soluble antigen complex with some of these procedures has had only limited success.

The development of a purified pertussis vaccine has been hampered by the modest yield of protective activity in the supernatant following simple salt extraction. On the other hand, the use of more aggressive agents to increase the yield of extracted protective antigen has produced apparently conflicting results, although some encouraging reports have emerged (7–10). In particular, the removal of the denaturing agent employed to extract the bacterial cells may cause at least some of the solubilized

material to precipitate (11), thus reducing the yield of protective activity. Furthermore, the complete removal of some detergent-type denaturants from solubilized protein may be difficult.

In the work presented here, we used polyacrylamide gel electrophoresis in SDS buffer to analyze the components brought into solution by various agents and to monitor their separation by conventional biochemical techniques. We also developed a procedure involving the use of a mineral carrier to adsorb the solubilized antigens before removal of detergent. This modification allowed large yields of protective antigen complex to be extracted from the bacterial cell surface for processing to a stable, adsorbed final product. We are now exploring the potential benefits of this material as an alternative to the conventional pertussis whole-cell vaccine.

MATERIALS AND METHODS

Chemicals

Ultrogel ® AcA 44 and Ampholine ® carrier ampholytes were obtained from LKB (Bromma, Sweden). Urea and deoxycholate were supplied by Merck (Darmstadt, Germany). Other detergents used were purchased from Serva (Heidelberg, Ger-

many). Reagents for polyacrylamide gel electrophoresis were also obtained from Serva.

Strains

Bordetella pertussis strain BB 114 was kindly supplied by Dr. C. R. Manclark, Bureau of Biologics, Bethesda, Maryland. It was kept in a lyophilized state, as was strain 41405, which was used in most experiments described in this report. For pilot experiments, the organisms were grown on slightly modified Cohen-Wheeler medium (12) in 15 liter New Brunswick fermentors fitted with an aeration device. The culture was started by adding 200 ml of exponentially growing organisms. The cells were harvested at 42–48 hours (final density corresponding to 20–40 $\times$ 10^9 organisms/ml) and resuspended in physiological saline in 5% of the original volume. Large scale preparations were performed in a similar fashion after fermentation on a 100–300 liter scale.

Extraction of Surface Components from Cell Concentrates

Sedimented *Bordetella pertussis* organisms were suspended in various media (Table 1) at a final concentration of 100 $\times$ 10^9 cells/ml and stirred for 60 minutes at 4°C. After centrifugation at 12,000 $\times$ g in a Sorvall SS–34 rotor, the supernatant was further centrifuged at 40,000 revolutions/minute in a

Table 1. Solubilization of Surface Antigens from *Bordetella pertussis*

Extracting Solvent	Yield of Solubilized Proteins	
	Strain BB 114	Strain 41405
	(μg/ml at 100 $\times$ 10^9 equivs./ml)	
1. None	30	28
2. 1 M NaCl	53	46
3. 2 M urea	63	80
4. 4 M urea	128	175
5. 6 M urea	243	283
6. 0.5% Deoxycholate	300	345
7. 1.0% Triton X–100	223	340
8. 1.0% Tween 20	83	140
9. 0.1 M Tris, pH 10.0	53	128
10. 0.5% Sodium dodecyl sulfate	650	830

All solvents used for extraction were prepared in 1 M NaCl–0.1 M Tris, pH 8.0. In solvent No. 9, the Tris buffer was adjusted to pH 10.0. The ultracentrifuge supernatants were dialyzed against water, lyophilized, and resuspended in 0.1 M KOH for Lowry protein determination (13). Alternatively, the extracted proteins were dialyzed against 4 M urea in 1 M NaCl–0.1 M Tris-HCl buffer, pH 8.0, and subjected to SDS gel electrophoresis (cf. Fig. 1)

Beckman L75 ultracentrifuge using rotor 50 Ti. The high-speed supernatants were collected and analyzed for protein (13,14) and by SDS gel electrophoresis (15). After preliminary experiments, the following procedure was adopted to prepare soluble extracts containing the protective antigen complex.

The bacteria were suspended in 6 M urea–1 M NaCl containing 0.1 M Tris–HCl buffer, pH 8.0 and stirred for 60 minutes at 4°C. The urea concentration was then adjusted to 4 moles/l by addition of 0.5 volume of 1 M NaCl–0.1 M Tris–HCl buffer, pH 8.0. After centrifuging as above, the supernatant was fractionated further (see below) or adsorbed to a mineral carrier consisting of 0.2% Al(OH)$_3$ and 0.15% AlPO$_4$ (final concentrations). After dialysis against physiological saline to remove the detergent, and after repeated washing of the adsorbed antigen complex, the protective potency of the preparation was estimated by established procedures (16).

Fractionation of Surface Components on Ultrogel ® AcA 44

Urea-salt extracts (50 ml, derived from a total of 5 $\times$ 10^{12} organisms) were dialyzed against 2 M urea–1 M NaCl, containing 0.1 M Tris-HCl buffer, pH 8.0, then concentrated 15-fold by ultrafiltration and applied to a column (2.5 $\times$ 100 cm) of Ultrogel ® AcA 44 equilibrated with the same buffer. Fractions (3 ml) were collected and analyzed at 280 nm. Appropriate pools were formed and subjected to SDS gel electrophoresis.

Fractionation of Surface Components by Isotochophoresis

Urea-salt extracts (50 ml; see above) were concentrated 10-fold and dialyzed against cathode buffer (30 g ε-aminocaproic acid, 1.5 g Tris, 240 g urea in 1,000 ml distilled water) for 18 hours at 4°C. Ampholine ® carrier ampholyte was added and the sample was subjected to preparative isotachophoresis in the LKB 7900 Uniphor apparatus. The manufacturer's instructions were followed, except that the gel column and all buffers contained 4 M urea to prevent the formation of aggregates during the separation. A current of 10–15 mA was maintained and fractions (2 ml) were collected at a rate of 15 ml/hour. To remove the carrier ampholytes, the pooled fractions were dialyzed for 5 days against 2 M urea–1 M NaCl containing 0.1 M Tris-HCl buffer, pH 8.0. The dialyzed fractions were analyzed directly or after ultrafiltration on SDS gel electrophoresis. Adsorption of the fractionated extract to Al(OH)$_3$–

AlPO$_4$ gel was performed as described above and was followed by dialysis against physiological saline.

RESULTS

Treatment of *Bordetella pertussis* organisms with several extracting agents followed by centrifugation to remove particulate matter resulted in the solubilization of highly variable amounts of protein material (Table 1). Whereas simple salt extraction was inefficient, the use of detergent mixtures such as urea or sodium deoxycholate increased the yield of protein in the supernatant by a factor of 5–10.

The corresponding patterns in SDS gel electrophoresis are shown in Figure 1. Under mild conditions, a single major component is recovered in the nonsedimentable fraction. In contrast, most samples prepared by detergent extraction reveal an extremely complex pattern involving a large number of protein zones (Fig. 1). In the experiments described below, material prepared by treatment of the cells with 6 M urea–1 M NaCl in 0.1 M Tris-HCl buffer, pH 8.0, was used.

About 15% of the protein material solubilized by the urea-salt procedure was sedimented by ultracentrifugation. Although the sediment appeared to contain some components also present in the supernatant, the patterns on SDS gel electrophoresis were not identical (Fig. 2). Most of the protective activity was recovered in the supernatant, with only marginal protective potency afforded by the corresponding particulate material (Table 2). Membrane filtration of the solubilized material reduced the final

yield of protective activity. Similarly, prolonged storage in 6 M urea was deleterious to the relevant antigen complex (Table 2). Therefore the extraction mixture was diluted to 4 M urea concentration immediately after the initial treatment of the bacteria with 6 M urea. At the reduced concentration of urea, the antigens were processed to the final adsorbed product without any apparent loss of activity due to protein denaturation. Typically, the extracted antigen, at a concentration corresponding to 100×10^9 organisms/ml, yielded protection values at least as high as those recorded for the whole cell vaccine at 30×10^9 organisms/ml. The cell residue remaining after detergent extraction had lost most of the protective potency (Table 3). Thus we concluded that extraction of the pertussis organisms with urea-salt solubilizes a substantial portion of the protective antigen complex and that the major portion of this complex remains in the supernatant following ultracentrifugation.

Fractionation of Surface Components by Gel Chromatography

Figure 3 shows the elution curve obtained on gel chromatography of solubilized antigens on a column of Ultrogel® AcA 44. Each fraction was electrophoretically analyzed on SDS polyacrylamide gels (inset, Fig. 3). This procedure allows the partial separation of several components present in the primary extract. Analysis of the protective activity revealed that animals receiving material eluting early from the gel column were more resistant to intracerebral challenge with virulent or-
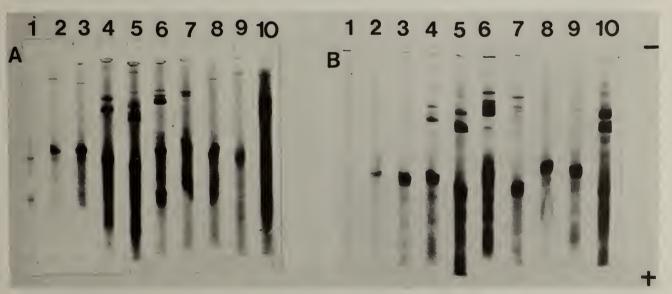
Figure 1. SDS gel electrophoresis of proteins solubilized with various agents. The extracting solvents, numbered 1–10, are described in Table 1. (A) Pattern derived from strain BB 114; (B) Pattern derived from strain 41405 (400×10^9 eq/ml).

Figure 2. SDS gel electrophoresis of material sedimentable by ultracentrifugation. A total of 5 × 10¹² cells were extracted with 6 M urea in 1 M NaCl–0.1 M Tris buffer, pH 8.0, as described in Materials and Methods. The resulting ultracentrifuge pellet was suspended in 10 ml of the same solvent and analyzed by SDS electrophoresis (Gel B, 25 μl applied) together with the corresponding ultracentrifuge supernatant (Gel A, 100 μl applied). Total yields: supernatant, 186 mg; pellet, 28 mg.

Table 2. Properties of *B. pertussis* Antigens Solubilized by Treatment with 6 M Urea–1 M NaCl at pH 8.0

Antigen Preparation	Extraction Time (hrs)	Equivalent Concentration of Adsorbed Extract[a]	Nitrogen Concentration of Adsorbed Extract (μg/ml)	Potency[b] IU/ml	Toxicity[c] (g/mouse) Day 3	Day 7
Urea-salt extract						
100,000 × g supernatant fraction	1	100 × 10⁹	70	12.3	+0.6	+5.4
100,000 × g supernatant fraction	20	100 × 10⁹	74	6.3	+0.2	+5.0
100,000 × g pellet fraction	1	100 × 10⁹	13	2.0	+1.5	+7.6
100,000 × g supernatant fraction, not filtered[d]	1	100 × 10⁹	77	16.7	+1.2	+3.6
100,000 × g supernatant fraction, filtered (0.2 mμ)[d]	1	100 × 10⁹	78	6.2	+1.1	+6.2

[a] Number of microorganisms corresponding to the concentration of the final product
[b] Mouse potency test (ref. 16)
[c] Mouse weight gain test (ref. 16)
[d] Data from a separate but similar experiment.

ganisms than mice previously immunized with low molecular weight components (Fig. 3).

Fractionation of Surface Components by Isotachophoresis

Figure 4 shows a characteristic pattern obtained by subjecting the dialyzed primary urea-salt extract to preparative isotachophoresis in buffer containing 4 M urea. The peaks were pooled as indicated and analyzed by SDS gel electrophoresis (inset, Fig. 4). Also shown are the survival rates following intracerebral challenge of animals given primary immunization with the fractions derived from the

isotachophoretic run. The material eluting in the later portion of the run consistently induced greater protection than the preceding fractions.

DISCUSSION

Ever since it became recognized that substances from the surface of *Bordetella pertussis* are involved in the elicitation of protective immunity, many reports have dealt with procedures to extract the protective antigen complex. Although some of the data seem to provide the basis for enthusiasm over the prospects of developing a purified, cell-free vac-

Table 3. Potency of Whole Cell *B. pertussis* Vaccine vs. Urea-Salt Solubilized Extract Antigen

Vaccine Type	Micro-oranisms (or equivalents) per ml	Potency [a] (IU/ml)	Toxicity [b] (g/mouse) Day 3	Day 7
Whole cell, adsorbed to mineral carrier	30×10^9	13.6	+1.3	+4.9
Extracted antigen, adsorbed to mineral carrier	100×10^9	15.3	+2.1	+7.4
Whole cell (residual material after treatment with urea-salt; adsorbed)	30×10^9	2.8	+2.6	+7.9

[a] Mouse potency test (16)
[b] Mouse weight-gain test (16).

cine against pertussis, it has been a frustrating experience to select a method yielding a final product with consistent properties. In addition to the problem of batch-to-batch variation, the low yield of protective antigen obtained by most procedures has presented substantial technological difficulties for large scale preparation. It is evident that the cost of a purified vaccine will be considerably higher than that of the conventional whole-cell product.

It is also clear that simple salt extraction of *Bordetella pertussis* does not result in an economically feasible yield of solubilized surface components. On the other hand, detergents, though effective in elevating the yield of solubilized material, seem to present problems of their own. Complete removal of the detergent may be difficult because of its noncovalent interaction with solubilized protein com-
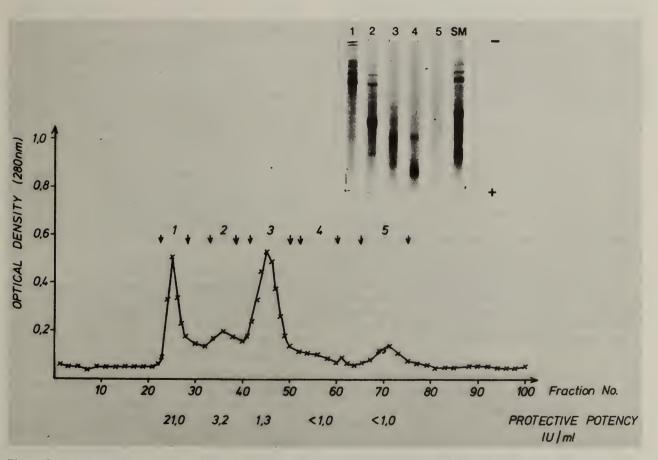
Figure 3. Gel chromatography of urea-salt extract of *Bordetella pertussis* on Ultrogel® AcA 44. Solubilized material from 5×10^{12} cells was applied to the column, which was eluted with 2 M urea in 1 M NaCl–0.1 M Tris buffer, pH 8.0. Fractions were pooled as indicated, concentrated by ultrafiltration, and analyzed by SDS gel electrophoresis. (Inset: the gel number corresponds to the respective pool; SM = starting material before application to the gel column). For analysis of the protective activity, each fraction was adsorbed to a mineral carrier as described in Materials and Methods and adjusted to a concentration equivalent to 200×10^9 microorganisms/ml.
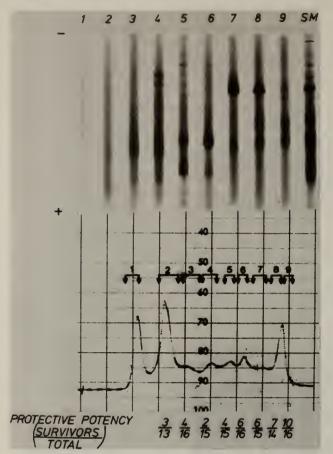
Figure 4. Preparative isotachophoresis of urea-salt extract of *Bordetella pertussis*. Solubilized material from 5×10^{12} cells was mixed with Ampholine® pH 7–9 carrier ampholyte and subjected to preparative isotachophoresis on an LKB 7900 Uniphor apparatus. Gel buffer No. 5 (Tris-phosphate buffer, pH 6.25) was used according to the manufacturer except that urea was added to a final concentration of 4 moles/l. Fractions were pooled as indicated and dialyzed extensively against 2 M urea–1 M NaCl in 0.1 M Tris-HCl buffer, pH 8.0, to remove the carrier ampholytes. (Inset: SDS gel electrophoretic analysis of each pool. SM = starting material for the preparative run.) To analyze for protective activity, vaccines were prepared by adsorption to a mineral carrier (see Materials and Methods). Each pool was tested as a single dose in groups of 16 animals, the total amount of antigen per animal corresponding to 1×10^9 microorganisms. The data are given in animals surviving challenge/total number of animals challenged.

ponents. Furthermore, even partial removal of the detergent might cause irreversible precipitation of part of the solubilized antigens (11).

The procedure described here helps circumvent some of these problems. A solution containing 6 M urea–1 M NaCl was selected as extracting agent because these solutes are easily removed by dialysis and apparently minimize the formation of aggregates. Also they produce a substantially higher yield of protective antigen complex than simple salt ex-

tractants do. It should be noted that a similar extraction procedure has been described by Lehrer et al. (11) for the preparation of the histamine sensitizing factor of *Bordetella pertussis*. The use of urea-salt mixtures to prevent the aggregation of the leucocytosis promoting factor isolated from culture supernatants has also been documented recently (17).

The large increase of solubilized material in the supernatant following detergent extraction and centrifugation was illustrated by the results of SDS gel electrophoresis (Fig. 1). Although much work is needed to characterize the different protein zones more fully, some preliminary observations may be made now. In simple salt extracts a single component dominates the SDS gel electrophoretic pattern, whereas under similar conditions detergent extracts contain many substances. Extracts showing an elevated protective potency in the mouse also seem to show considerably more migrating protein zones in the molecular weight range above 70,000.

Application of SDS gel electrophoresis to the surveillance of the routine preparation of solubilized pertussis antigen complex may be worth exploring. In analyzing cell envelope proteins from *Bordetella pertussis* by SDS gel electrophoresis, Wardlaw et al. (18) have recently described characteristic differences in the patterns derived from phase I and phase IV organisms as well as changes induced by varying growth conditions. Except for one experiment involving growth in the presence of sodium succinate (18), the loss of two envelope polypeptides with molecular weights of about 28,000 and 30,000, respectively, was well correlated with a diminished protective activity of the corresponding whole cell vaccine.

The electrophoretic pattern from the cell envelope fraction (18) seems even more complicated than that derived from the surface antigens solubilized by urea-salt extraction (Fig. 1). Therefore, preparations of the latter type might offer some advantages in attempting to define the protein zones that appear to be associated with protection against pertussis. The application of preparative isotachophoresis in the presence of urea seems to be a promising approach for future attempts to resolve the components of the primary extract. Clearly, SDS gel electrophoresis may be of considerable value in monitoring the fractionation achieved by this or other separation techniques.

Adsorption of the detergent-solubilized components of *Bordetella pertussis* to a mineral carrier

before removal of denaturing agent avoided aggregation and loss of antigenic material. When adjusted to similar potency in the mouse protection test, the adsorbed antigen mixture contains less protein nitrogen and appears to be less reactogenic than the conventional whole cell vaccine from which it was derived. The product containing adsorbed surface antigens from *Bordetella pertussis* may thus be a suitable alternative to the cellular vaccine currently in use.

Conceivably, the urea-salt extract may also be an appropriate starting material for further purification of the protective antigen. In agreement with numerous other studies, our experience indicates that certain fractions of the primary extract may be associated with a higher level of protection in the mouse potency test, whereas other fractions are less active (Figs. 3 and 4). However, fractionation invariably reduces the total yield of protective activity as measured by the mouse potency test. Furthermore, the mouse potency test is too complicated to safely evaluate the large number of samples produced in almost any separation scheme. Therefore, efforts to develop a simple in vitro assay for the antigen(s) involved in the protection of humans against disease should be continued. Until a new, less complicated method that allows for alternative definition of the relevant antigen complex becomes available, efforts to arrive at purified pertussis vaccines may have to be limited to the preparation of the primary bacterial extract. Finally, high priority should be given to further studies on the effects of various production strains and growth conditions on the potency and reactogenicity of the final product.

REFERENCES

1. Munoz, J. J. Symposium on relationship of structure of microorganisms to their immunological properties. I. Immunological and other biological activities of *Bordetella pertussis* antigens. Bacteriol. Rev. 27:325–340; 1963.

2. Pittman, M. *Bordetella pertussis:* bacterial and host factors in the pathogenesis and prevention of whooping cough. Mudd, S., ed. Infectious agents and host reactions. Philadelphia, Pa: Saunders; 1970:239–270.

3. Morse, S. I. Biologically active components and properties of *Bordetella pertussis*. Adv. Appl. Microbiol. 20: 9–26; 1976.

4. Pennel, R. B.; Thiele, E. H. Studies on the fractionation of *Hemophilus pertussis* extracts. J. Immunol. 66:627–633; 1951.

5. Munoz, J.; Hestekin, B. M. Antigens of *Bordetella pertussis*. III. The protective antigen. Proc. Soc. Exp. Biol. Med. 112:799–805; 1963.

6. Dahlstrom, R. V. Pertussis antigen. U.S. patent 3,141,824 assigned to Eli Lilly and Company, Indianapolis, Ind., July 21, 1964.

7. Maitland, H. B.; Guérault, A. Some surface components of *Haemophilus pertussis:* Immunizing antigen, histamine-sensitizing factor and agglutinogen. J. Pathol. Bacteriol. 76:257–274; 1958.

8. Barta, G. Soluble protective antigen from *Bordetella pertussis* prepared with sodium desoxycholate. J. Immunol. 90:72–80; 1963.

9. Sutherland, I. W. The protective activity of components of *Bordetella pertussis* cell walls. Immunology 6:246–254; 1963.

10. Nagel, J. Isolation from *Bordetella pertussis* of protective antigen free from toxic activity and histamine-sensitizing factor. Nature 214:96–97; 1967.

11. Lehrer, S. B.; Tan, E. M.; Vaughan, J. H. Extraction and purification of the histamine-sensitizing factor of *Bordetella pertussis*. J. Immunol. 113:18–26; 1974.

12. Cohen, S. M.; Wheeler, M. W. Pertussis vaccine prepared with phase I cultures grown in fluid medium. Am. J. Public Health 36:371–376; 1946.

13. Lowry, O. H.; Rosebrough, N. J.; Farr, R. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275; 1951.

14. Spector, T. Refinement of the Coomassie Blue method of protein quantitation. A simple and linear spectrophotometric assay for 0.5 to 50 μg of protein. Anal. Biochem. 86:142–146; 1978.

15. Weber, K.; Osborn, M. The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406–4412; 1969.

16. Additional standards: pertussis vaccine. Federal Register 33:8818–8819; 1968.

17. Morse, S. I. Lymphocytosis-promoting factor of *Bordetella pertussis:* isolation, characterization and biological activity. J. Infect. Dis. 136:S234–S238; 1978.

18. Wardlaw, A. C.; Parton, R.; Hooker, M. J. Loss of protective antigen, histamine-sensitizing factor and envelope polypeptides in cultural variants of *Bordetella pertussis*. J. Med. Microbiol. 9:89–100; 1976.

Substrate Specificity and the Purification by Affinity Combination Methods of the Two *Bordetella pertussis* Hemagglutinins

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ABSTRACT

The substrate specificities of the leukocytosis promoting factor hemagglutinin and the fimbrial hemagglutinin of *Bordetella pertussis* were investigated by erythrocyte agglutination methods. A simple, one-step affinity combination method for the purification of the leukocytosis promoting factor has been devised based on an identification of its specificity for a sialic acid-containing receptor. The specificity of the fimbrial hemagglutinin for multilamellar model membranes (liposomes) containing cholesterol has been utilized to achieve its purification. Data on the immunological and protective properties of the hemagglutinins are presented with special reference to a novel infection/protection model in the rabbit.

INTRODUCTION

Cells of *Bordetella pertussis* phase I strains can produce two distinct hemagglutinins (1). One of them, leukocytosis promoting factor—hemagglutinin (LPF–HA), is produced under various conditions of culture aeration and can induce a complex of biological responses, including lymphocytosis, histamine sensitization, and mitogenic and adjuvant effects. The second hemagglutinin, fimbrial hemagglutinin (F–HA), appears to be associated with the fimbriate state (1,2) and its production is greatly favored in static, poorly aerated cultures. Studies on the properties of *B. pertussis* hemagglutinin that predate the recognition of these two distinct hemagglutinins are difficult to interpret.

This report describes our simple, one-step method for the purification of LPF–HA based on an identification of its specificity for a sialic acid-containing receptor, and suggests how the specificity of the second hemagglutinin, F–HA, for cholesterol (3,4) can be utilized to achieve its purification. Data on the immunological and protective properties of the hemagglutinins are also presented, with special reference to a novel infection/protection model in the rabbit.

MATERIALS AND METHODS

Chemicals

Apart from general reagents for buffers, etc., or as otherwise noted, chemicals used were Sigma products. The neuraminidases used were from *Clostridium welchii* (Worthington) or from *Vibrio cholerae* (crude culture filtrate from Wellcome Laboratories, or purified BDH enzyme). Purified haptoglobin was prepared from a single type 2:2 donor's plasma by the method of Connell and Shaw (5). This preparation gave a major arc of precipitation on immunoelectrophoresis with rabbit antiserum to whole human serum (Hoechst Pharmaceuticals) and two minor arcs, one of which was due to albumin. It gave a single intense arc with specific rabbit antiserum to human haptoglobin. Purified cholera toxin was a gift from Dr. R. A. Finkelstein, Department of Microbiology, University of Texas. The GM_1 monosialyl ganglioside was a "Supelco" preparation, obtained from Chromatographic Product Services Ltd., Wirral, Merseyside, U.K. Egg yolk lecithin was obtained from Lipid Products Ltd., U.K.

Strains of Bacteria

The 1.2 serotype strain Tohama and the 134 strain were obtained from Dr. Jean Dolby, The Lister Institute, Elstree, Herts., U.K., and the 1.3 serotype strain, M2, from Dr. N. Preston, Department of Bacteriology, University of Manchester, U.K. The BB 114 (3779B) strain was obtained from Dr. C. R. Manclark, Bureau of Biologics, Bethesda, Maryland.

Growth of Bacteria

Three-day reciprocating shaker growths in Thompson bottles were made in Cohen and Wheeler medium (6). A modified medium (2) was used for 5-day growths, whether shaken or static.

Cell Disintegrates

Cell suspensions disintegrated mechanically (Dyno Mill, W. A. Bachofen, Basel, Switzerland) or by sonication (Dawe Soniprobe, maximum amplitude, 20 minutes with cooling intervals) were centrifuged at 10,000 × g for 30 minutes. Supernatants generally contained more than 20 mg protein/ml or were adjusted by pressure dialysis to about this level.

Hemagglutination Methods

Sheep, chicken, and goose blood was collected into Alsevers solution and used within 7 days. Cells were washed three times in 20 volumes of phosphate-buffered saline, pH 7.2, by centrifugation. The cells were finally used at 0.5% (v/v) suspensions in either Microtiter or tube agglutination tests, being mixed with an equal volume of doubling dilutions of the test substance. Tests carried out in tubes were read after 16 hours at 4° by pattern and by "flicking." Microtiter tests were recorded after 2 hours at room temperature.

Titers are uniformly expressed as the reciprocal of the final dilution in the hemagglutinin/erythrocyte mixture. Where not otherwise noted, goose cells were used.

In inhibition tests (tube only), the inhibitor dilutions (0.1 ml) were treated with an equal volume of a hemagglutinin preparation that was first adjusted to a sixteenfold dilution from its hemagglutination endpoint. After mixing and incubation at room temperature for 1 hour, 0.2 ml of erythrocyte suspension was added and mixed. The mixture thus finally contained "four hemagglutinating doses" of hemagglutinin, a condition under which inhibition could be sensitively determined. Tests were read after 16 hours at 4°. Inhibition titers are expressed as μg/ml of inhibitor in the final inhibitor/hemagglutinin/erythrocyte mixture.

Hemagglutinins

B. pertussis hemagglutinins were either crude or purified preparations as detailed in the text. The influenza hemagglutinin was a crude virus suspension from an egg growth of the BEL strain, kindly prepared and tested by Dr. G. Appleyard.

Immunodiffusion

A conventional Ouchterlony double diffusion technique was used.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) was carried out in 0.7 × 7.8 cm rods (7). Proteins were dissociated by heating in 1% (w/v) SDS–1% (v/v) 2-mercaptoethanol at 100° C for 5 minutes. Electrophoresis was also carried out in 5% acrylamide gels without SDS at pH 4.3 by the method of Reisfeld (8). Gels were stained with Coomassie Blue R250 (BDH) after electrophoresis.

Biological Assays

Leukocytosis promoting and histamine sensitizing activities were determined by the method of Morse and Morse (2).

RESULTS

As detailed below, our studies demonstrate that LPF–HA and F–HA can be readily distinguished from each other by the physical character of the erythrocyte agglutination they produce. LPF–HA produces coherent sheets of cells; F–HA simply influences the settling pattern of the cells. This distinction can be made only in tube agglutination tests, where the effects of shearing forces on settled cells can be observed by "flicking." It is concealed in the Microtiter assays generally employed (1,2). Thus even in crude preparations containing both LPF–HA and F–HA the dominant hemagglutinin alone is expressed in the higher dilutions of a hemagglutination titration and its properties can be studied even in the presence of a minor proportion of the other hemagglutinin. In the rest of this report crude and purified preparations are distinguished by the notations LPF–HA (crude) and LPF–HA respectively.

Receptor Specificity of LPF–HA

LPF–HA is selectively expressed in cell disintegrates (Dyno Mill or sonicate) obtained from 3- to 5-day shake cultures of *B. pertussis*. The influence of neuraminidase on different aspects of the hemagglutinating activity of such extracts is illustrated in Tables 1 and 2.

Table 1 shows that neuraminidase treatment of red cells does not diminish their agglutination by LPF–HA (crude), in agreement with the observations of others (2) and in distinction from its effect on the action of the influenza virus hemagglutinin (9,10). However when the cells as used in Table 1 are incubated with a standard dose of LPF–HA (crude), the enzyme-treated cells are much less able to adsorb the hemagglutinin than are untreated cells, as measured by the ability of supernatant fluids from these mixtures to agglutinate untreated goose erythrocytes. It is important for the interpre-

Table 1. The Agglutination of Untreated and Neuraminidase-Treated Avian and Mammalian Erythrocytes by the Hemagglutinin of Influenza Virus and the LPF–HA (Crude) of *B. pertussis*

| Erythro-cytes | Titer | | | | | |
| | Influenza hemagglutinin | | | *B. pertussis* LPF–HA (crude) hemagglutinin | | |
	Un-treated	Crude enzyme	Purified enzyme	Un-treated	Crude enzyme	Purified enzyme
Goose	2560	<20	<20	320	640	640
Chicken	2560	<20	<20	40	40	40
Sheep	480	—	<20	40	—	40

2.5% v/v suspensions of washed red cells in saline containing 10mM $CaCl_2$ were adjusted to pH 6.0 at 40°. One 10 ml volume was centrifuged and the cells resuspended in 4 ml of Wellcome crude "receptor destroying enzyme." Other 10 ml volumes received 100 ml of purified BDH *V. cholerae* neuraminidase or were untreated. After 2 hours at 37° the cells were centrifuged off and treated with fresh enzyme for another 2 hours, then recentrifuged and suspended in 50 ml phosphate buffered saline pH 7.2 (0.5% v/v). Hemagglutination was measured by a tube test.

tation of this experiment to note that enzyme treatment did not significantly alter the red cell count with respect to untreated preparations. In this respect the *V. cholerae* enzyme differs from that of *C. welshii,* which has lytic properties.

Inhibition of LPF–HA by serum proteins. Normal sera from man, marmoset, vervet, and rabbit were inhibitory in hemagglutination tests at dilutions up to a hundredfold. The Cohn alcohol fractions of human serum (Miles Laboratories) were also inhibitory in the following order of decreasing activity: α-globulins (20 μg/ml) > glycoprotein >> β-globulin > γ-globulin > albumin (inactive at 1 mg).

Individual sialoproteins were then tested and found to have different inhibitory activities. Haptoglobin and ceruloplasmin Type III were active at 1–10 μg/ml. Bovine submaxillary mucin and human bronchial mucin (the latter, from Dr. G. Roberts, The Welsh National School of Medicine, Cardiff, U.K.) were active, but less so. Transferrin, conalbumin types I and II (with and without Fe), lactoferrin, and ferritin were inactive at 1 mg/ml. The commercial source of the products was important. Transferrin from one source was heavily contaminated with haptoglobin, as measured by immunodiffusion with specific haptoglobin antiserum, and was misleadingly active in inhibition tests. The activity of ceruloplasmin Type III was shown by this test not to be due to haptoglobin contamination.

Although the order of inhibition by the substances listed did not vary from test to test, actual values were rather erratic in tests performed many times over a period of 1 year. The age of the red cell preparations (1–5 days), and the thoroughness of washing were important variables, but the pH and incubation times were much less so.

For all the substances listed above where inhibition was observed, it could be reduced to less than 1% of its value by prior treatment of the material with neuraminidase. For this purpose samples of test substance at 10 mg/ml in 0.1 M acetate buffer, pH 5.0, were treated with *C. welchii* neuraminidase at a final concentration of 200 μg/ml. A control preparation in acetate buffer received diluent alone at this stage. Experimental and control preparations were dialyzed against 100 volumes of 0.1 M acetate buffer, pH 5.0, at 37° for 4 hours, then against 100 volumes of isotonic phosphate-buffered saline, pH

Table 2. The Agglutination of Goose Erythrocytes by LPF–HA (Crude) of *B. pertussis* After Adsorption by Untreated and Neuraminidase Treated Avian and Mammalian Erythrocytes

| Erythrocytes | Titer | | | | | | | | |
| | Cells (neat) [a] | | | Cells (1/3) | | | Cells (1/9) | | |
	Untreated	Crude enzyme	Purified enzyme	Untreated	Crude enzyme	Purified enzyme	Untreated	Crude enzyme	Purified enzyme
Goose	<2	8	8	2	8	8	8	16	16
Chicken	<2	8	8	4	8	16	8	16	16
Sheep	2	—	8	4	—	8	8	—	16

[a] Neuraminidase-treated or untreated erythrocytes (see Table 1) were centrifuged and resuspended in 1/20 the original volume of phosphate buffered saline. Samples of this suspension and threefold and ninefold dilutions of it were each incubated with an equal volume of LPF–HA (crude) having an agglutination titer of 1/32. The suspensions were then centrifuged and the supernatants titrated against fresh goose cells in a tube test.

7.2, at 4° for a further 16 hours. The dialysis sac contents were heated at 70° for 30 minutes to destroy hemolytic activity and tested in parallel against red cells for residual hemagglutinin activity.

All the results described above were obtained with LPF–HA (crude). They were confirmed with LPF–HA when it became available from the purification procedure described below. Table 3 illustrates results obtained with LPF–HA for enzyme-treated and untreated serum proteins. It also shows that of eight sugars tested, only N-acetylneuraminic acid and neuraminyl lactose were inhibitory. Although inhibition was weak, it was reproducible. D-glucuronic acid and cholesterol liposomes (see below) were included in this test because of earlier claims (3,11) that they contributed to the receptor specificity of pertussis hemagglutinin. We conclude that they do not contribute to LPF–HA receptor specificity.

Finally, with respect to LPF–HA, the availability of pure material allowed direct visualization of its reactivity with haptoglobin as a band of precipitation in an immunodiffusion test (Fig. 1). Figure 1 shows that the specificity of LPF–HA for sialo-compounds differs from that of cholera toxin since only the latter precipitates with the GM_1 monosialyl ganglioside (12) (moreover, this compound does not inhibit LPF–HA in hemagglutination tests at 1 mg/ml). On other plates, it was shown that serum glycoproteins and submaxillary mucin resemble haptoglobin in precipitating with LPF–HA.

Purification of LPF–HA by Affinity Chromatography

The specific binding of LPF–HA to haptoglobin is the basis of an affinity chromatography method for purification of LPF–HA (U.K. Patent Application 8089/78). The method, which will be reported in full detail elsewhere (Irons and MacLennan, to be published), involves the following steps.

1. Coupling purified human haptoglobin (Type 2:2) to CNBr-activated Sepharose 4B (Pharmacia Ltd.).
2. Applying to the column extracts of *B. pertussis* cells disintegrates in 50 mM phosphate–0.5M NaCl pH 6.5 buffer. About 50% of the applied hemagglutinin activity is bound to the adsorbent, which is then washed with about 20 column volumes of buffer to remove unbound material.

Table 3. The Inhibitory Action of Sialoproteins and Neuraminyl Sugars on the Agglutination of Goose Erythrocytes by Purified LPF–HA

Test Substance	Hemagglutination									
	μg inhibitor/ml in final inhibitor/hemagglutination/red cell mixture									
	5000	2500	1250	625	312	156	78	39	20	10
Haptoglobin [a]	0	0	0	0	0	0	0	±	2	3/A
Haptoglobin [a] (neuraminidase-treated)	3	3	3	3	4	4	4	4	4	4
Ceruloplasmin	0	0	0	0	0	0	2	3/A	A	A
Transferrin	3	4	4	4	3	3	3/A	A	A	A
Albumin	3	4	4	4	4	3	3	3	3	A
L–Fucose	A	A	A	A	A					
D–Galactose	A	A	A	A	A					
D–Mannose	A	A	A	A	A					
D–Glucuronic acid [b]	A	A	A	A	A					
N-Acetyl neuraminic acid [b]	1	2/A	3/A	3/A	A					
Neuraminyl lactose [b]	3	3/A	A	A	A					
N-Acetyl-glucosamine	A	A	A	A	A					
N-Acetylgalactosamine	A	A	A	A	A					
Cholesterol liposomes [c]		Not tested		A	A					

The LPF–HA preparation had the properties described in the text for purified LPF–HA.

The symbols 0–4 denote the level of agglutination from free cells to a coherent sheet. The letter A denotes firm adherence of the cells as a fine film to the bottom of the tube; it is an expression of 4 agglutination in the absence of protein that prevents adherence.

[a] The conditions for neuraminidase treatment and the treatment of the control are detailed in the text.

[b] Solutions were adjusted to approximately pH 7.0 by addition of alkali.

[c] For preparation, see text.

Figure 1. Specificity of precipitation reactions of cholera toxin and LPF–HA with sialo compounds. LPF: affinity chromatography purified LPF–HA, 0.25 mg/ml; GM: purified human GM1 ganglioside, 2.5 mg/ml; HP: purified human haptoglobin, 1 mg/ml; CT: purified cholera toxin, 1 mg/ml; AS: rabbit antiserum to partially purified LPF; photographed after 48 hours.

Table 4. Biological Activities of LPF–HA Isolated by Affinity Chromatography

Specific hemagglutinin activity [a]	60,000–120,000 U/mg (goose cells) 6,000–8,000 U/mg (chicken cells)	
LPF activity [b]	μg injected/mouse	WBC$\times 10^3$/mm^3
	zero	3.4
	0.02	8.5
	0.1	5.9
	0.5	10.0
	1.0	19.5
	2.0	35.9
Histamine sensitizing activity [c]	0.03–0.05 μg	

[a] Hemagglutination measured by a Microtiter method
[b] Groups of 5 CF1 mice were injected intravenously and white blood cell counts performed 3 days later.
[c] 50% sensitizing dose for histamine lethality in NIH (Dubby strain) mice

3. Eluting LPF–HA activity by a stepwise change to 0.1 M Tris–0.5 M NaCl pH 10 buffer. The active eluate is concentrated by vacuum dialysis against 50 mM Tris–1 M NaCl pH 8 buffer to about 300–400 μg protein/ml.

The cell extract used for this preparation was obtained from the Phase I Tohama strain of *B. pertussis,* grown aerobically in Cohen and Wheeler medium at 37° for 3 or 5 days. After the cells were separated from the medium, they were suspended in 0.01 M Tris–0.145 M NaCl–5 mM MgCl$_2$–15 mM NaN$_3$ buffer pH 7.4 at a concentration of about 12% (w/v) and disintegrated in the Dyno Mill. The cells were removed by centrifuging at 10,000 × g for 30 minutes and the supernatant was again centrifuged at 360,000 × g for 90 minutes. Ammonium sulfate was added to the supernatant to 74% saturation and the precipitate was extracted several times with small volumes of 50 mM Tris–1 M NaCl pH 8.0 buffer. Extracts containing hemagglutinin activity were pooled and dialyzed against the pH 8 Tris–NaCl buffer. These extracts had a specific hemagglutinating activity of about 200 U/mg when measured by a Microtiter test using goose erythrocytes and a protein concentration of about 40 mg/ml.

The specific hemagglutinin activity of the LPF–HA eluted from the haptoglobin–Sepharose conjugate was about 60,000–120,000 U/mg and 6,000–8,000 U/mg when measured with goose and chicken erythrocytes respectively (Table 4). This represents a 300 to 600-fold purification of the hemagglutinin from the extract and about 1,000-fold purification

from the supernatant obtained after the initial cell disintegration and centrifugation. About 1–2 mg of purified LPF–HA was obtained from 30 ml of cell extract using a 1 × 10 cm column of adsorbent.

The LPF–HA obtained by affinity chromatography was examined by polyacrylamide gel electrophoresis (PAGE). In 11.7% acrylamide gels (acrylamide/bis ratio 76/1) in the presence of sodium dodecyl sulphate (SDS) it showed four major bands and a few faint minor ones (Fig. 2). The molecular weights of the major bands measured in 12.5% acrylamide rods (acrylamide/bis ratio 37/1) were 12,600, 21,100, 22,400 and 27,200. Electrophoresis in 5% acrylamide gels at pH 4.3 gave a major but rather diffuse band (Fig. 2) with a mobility relative to the Pyronin Y tracker dye of 0.52. Electron microscopy of the LPF–HA in Tris-NaCl pH 10 buffer showed the predominant structure as roughly spherical particles about 60Å in diameter. These tended to form aggregates so that individual structures could not be clearly seen.

The biological properties of the LPF–HA are shown in Table 4. It was a potent inducer of leukocytosis; injection of 0.02 μg in CF–1 mice was followed by a twofold increase in total white blood cell counts, measured 3 days later. It also had a high histamine sensitizing activity in NIH (Dubby strain) mice; injection of 0.03–0.05 μg sensitized 50% of the mice to the lethal effect of 1 mg histamine. Pronounced adjuvant activity was also present; intraperitoneal injection of 0.01 μg together with ovalbumin into mice enhanced the antibody response to ovalbumin. The biological activities of the LPF–HA purified by affinity chromatography shown in Table 4 are very similar to those reported

This method of purification is much simpler than the various chromatographic methods used in the past (13,14) and gives a much more homogeneous product. The isolation of LPF–HA from cell disintegrates is particularly difficult by conventional separative methods since it constitutes less than 1% of the total proteins present. Also, purification methods using long columns of agarose gels lead to a loss of LPF–HA activity by direct interaction of the molecule with the agarose matrix (1). This interaction is no problem with the very small columns of agarose gel used in affinity chromatography.

Although the present method of preparation has mainly utilized extracts from 3- and 5-day growths of the Tohama strain, it has also worked with the BB 114 (3779B) strain. However, less satisfactory products have been obtained from the M2 strain. The reason for this difference is not known at present.

Receptor Specificity of F–HA

Early work by Fisher (3) on the specificity of the interaction of *B. pertussis* hemagglutinins with human red cell membranes suggested that cholesterol might be important for this interaction. This has recently been confirmed by Sato (4). In this paper we present some recent results on the interaction of F–HA with model membranes (liposomes) containing cholesterol and other sterols and show how this interaction can be used as a possible method of purification.

For this work cells of the phase I BB 114 (3779B) strain of *B. pertussis* were grown in shallow, static cultures in a liquid medium (2). After 5 days at 37° C the cells were centrifuged off and ammonium sulfate added to the culture supernatant to 74% saturation. The precipitate was extracted 10–12 times with small volumes of 50 mM Tris–1 M NaCl pH 8.0 buffer. Extracts containing hemagglutinin activity were pooled and centrifuged at 360,000 × g for 90 minutes, and the supernatant was concentrated to about 1 mg/ml of protein. When examined by SDS–PAGE this extract showed a complex pattern (Fig. 3), but the most striking feature of the pattern was the presence of two intense bands near the stacking gel, with molecular weights of about 127,000 and 95,000. These bands could be removed from the extracts by treatment with human erythrocyte stroma with a concurrent removal of 95% of the hemagglutinin activity. These proteins could also be partly separated from other proteins present in the extract by gel filtration at

Figure 2. Polyacrylamide gel electrophoresis of LPF–HA. Left, in 5% acrylamide gels at pH 4.3. Right, in 11.7% acrylamide gels with SDS.

by Morse and Morse (2), and the specific hemagglutinin activity measured with chicken erythrocytes is identical to that found by Arai and Sato (1). Moreover, the subunit molecular weights found by SDS–PAGE are close to those reported by Morse and Morse (2). These workers found that SDS-mercaptoethanol-urea dissociated LPF–HA into four subunits of about equal intensity, while Arai and Sato (1) found their LPF–HA produced two major and two minor bands of molecular weights 26,000, 20,000, 13,000, and 10,000 respectively.

The LPF–HA isolated by affinity chromatography from cell disintegrates appears to be about as pure as that isolated by other workers (1,2) from culture supernatants obtained after maximum growth of cells. It is difficult to quantitatively measure the degree of contamination of our preparation by minor components, but densitometric scanning of SDS–PAGE gels suggests the preparation is around 90–95% homogeneous. Immunodiffusion with specific antiserum (below) reveals contamination with agglutinogen 2 of about 5%, and at least one other antigen is also present.

Figure 3. SDS–PAGE of *B. pertussis* cell extracts after treatment with different liposomes: Sample 1: Original cell extract. The arrows indicate proteins of molecular weight 127,000 and 95,000. Samples (left to right) 2 and 3, 4 and 5: liposome suspensions 6 and 7, 8 and 9 at 26,000 × g for 1 hour. Samples (left to right): supernatants obtained after mixing cell extracts with liposomes composed of (6) EYL/DCP; (7) EYL/DCP/epicoprostonal; (8) EYL/DCP/cholesterol; (9) EYL/DCP/dihydrocholesterol.

4° C on a 2.5 × 84 cm column of Sepharose 6B. When examined in the electron microscope the chromatographically purified proteins had the appearance of fine filaments about 20 Å in diameter but of variable length (300–500 Å). Comparison of these data with the results of Arai and Sato (1) suggests that the proteins in our extracts responsible for the characteristic SDS–PAGE bands probably represent native or partly degraded forms of the fimbrial hemagglutinin, F–HA.

The interaction of F–HA present in these cell extracts with liposomes containing various sterols was studied using multilamellar liposomes prepared by standard procedures from mixtures of 14.7 mM egg yolk lecithin (EYL), 2.9 mM dicetyl phosphate (DCP) and 14.7 mM sterol (molar ratio 5/1/5). After removal of chloroform from the lipid mixtures, 4 ml of 50 mM Tris–1 M NaCl pH 8 buffer was added to the dried lipid film and liposomes allowed to form by standing at room temperature under nitrogen for about 6 hours with occasional hand shaking. One ml of the liposome suspension was mixed with 1 ml of the cell extract containing F–HA activity and the mixture allowed to stand at 4° overnight. The liposomes were removed from the suspension by centrifuging at 26,000 × g for 1 hour at 4° so that they formed a surface layer on the buffer solution. The solution

recovered from beneath this layer was used for the measurement of hemagglutinin activity, and both this solution and the liposome layer were used for SDS–PAGE studies.

The results of an experiment comparing liposomes composed of egg yolk lecithin, dicetyl phosphate, and cholesterol, dihydrocholesterol, or epicoprostanol with liposomes containing only egg yolk lecithin and dicetyl phosphate are shown in Table 5 and Figure 3.

The results of the hemagglutinin assay suggest that only liposomes containing cholesterol or dihydrocholesterol will remove F–HA from the cell extract. This is confirmed by the SDS–PAGE patterns, which show removal of the F–HA bands by these liposomes and not by liposomes containing epicoprostanol or dicetyl phosphate. Further confirmation of this effect is shown by direct examination by SDS–PAGE of the liposomes recovered from the cell extract by centrifugation (Fig. 3).

A number of other sterols including ergosterol, lanosterol, β-sitosterol, and stigmasterol have been examined by this procedure. The results show that despite the similar chemical structures of these sterols only liposomes containing cholesterol, dihydrocholesterol, or β-sitosterol will interact with F–HA.

Purification of F–HA by an Affinity Combination Method

We attempted to elute bound F–HA from liposomes as a possible "affinity" method of purification. For these experiments liposomes containing bound F–HA were suspended in small volumes of various buffer solutions at 4° C overnight and centrifuged at 26,000 × g for 1 hour. The supernatant was tested for hemagglutinin activity. The results (Table 6) show that little hemagglutinin activity is eluted by phosphate or Tris buffers of pH 7–10: However, addition of 1–2 M concentrations of the

Table 5. The Adsorption of F–HA Activity from F–HA (Crude) by Treatment with Liposomes Containing Different Sterols

	Titer [a]
Extract	256
Supernatants after treatment with:	
EYL/DCP/cholesterol liposomes	32–64
EYL/DCP/dihydrocholesterol liposomes	32
EYL/DCP/epicoprostanol liposomes	256–512
EYL/DCP liposomes	128–256

[a] Hemagglutination was measured by a Microtiter method.

Table 6. Elution of F–HA Activity from F–HA Liposome Complexes by Different Buffer Solutions

	Titer [a]
Extract	4096–8192
Supernatant after liposome treatment	
Eluting buffer:	256
50 mM phosphate — 0.5 M NaCl, pH 7	4
50 mM Tris — 1 M NaCl, pH 8	4
0.1 M Tris — 0.5 M NaCl pH 10	16
Tris — NaCl, pH 8 + 1 M KI	4
Tris — NaCl, pH 8 + 2 M KI	16
Tris — NaCl, pH 10 + 1 M KI	32–64
Tris — NaCl, pH 10 + 2 M KI	256
Tris — NaCl, pH 10 + 1 M KSCN	1024–2048
Tris — NaCl, pH 10 + 2 M KSCN	256–512

[a] Hemagglutination was measured by a Microtiter method.

chaotropic salts KI or KSCN to the pH 10 buffer releases about 30–60% of the bound F–HA in an active form. SDS–PAGE of the F–HA eluted by 0.1 M Tris–0.5 M NaCl–1 M KSCN pH 10 buffer (Fig. 4) shows that it has the characteristic F–HA bands with molecular weights of 127,000 and 95,000, as well as several other components. The specific hemagglutinin activity of F–HA eluted from liposomes by this procedure has been found to be about $1 \times 10^5 - 3 \times 10^5$ U/mg when measured with goose erythrocytes, which is similar to that found for F–HA purified by Sepharose 6B chromatography. However, the hemagglutinin activity of the eluted F–HA has been found to decrease rapidly on storage at 4° C, indicating that the molecule is not very stable.

Figure 4. SDS–PAGE of F–HA eluted from liposomes by 0.1 M Tris–0.05 M NaCl–1 M KSCN pH 10 buffer. Samples (left to right): (1) Tris–NaCl–KSCN eluate of liposomes containing bound F–HA; (2) supernatant obtained after mixing cell extract with EYL/DCP/cholesterol liposomes; (3) cell extract. The arrows indicate proteins of molecular weight 127,000 and 95,000.

This work suggests that the interaction of F–HA with liposomes containing cholesterol might be a useful "affinity" method of purifying F–HA. The homogeneity of the material obtained by this method is similar to that obtained by gel exclusion chromatography, although the nature of the minor components shown on SDS–PAGE (Fig. 4) needs investigation. The present work shows that a sterol with a chemical structure very close to that of cholesterol is needed in the liposomal bilayer structure for the interaction with F–HA to occur. However, comparison of the effects produced by the different sterols suggests that a steric combination of phospholipid head group and the correct sterol hydroxyl group might be the binding site on the membrane surface for F–HA. Further work is in progress to establish the chemistry of this interaction and to find alternative methods of elution.

The Physical Character of Hemagglutination by LPF–HA and F–HA

The LPF–HA and F–HA hemagglutinins, purified as described above, produce agglutination of erythrocytes in both Microtiter and tube assays. When titer is read by the pattern of settled cells there is no obvious difference in the effect produced on red cells by the two substances. However, if the cell sediment is resuspended by flicking, which can only be done readily in the tube test, a pronounced difference is apparent. Red cells agglutinated by LPF–HA are found to be firmly bound into coherent sheets, which do not disperse on flicking even a few dilution steps from the endpoint, while cells agglutinated by F–HA are dispersed largely into a single cell suspension by even the gentlest flicking. This property allows the measurement of LPF–HA to be made in the presence of an excess of F–HA by employing a tube test in which a "pattern" reading is followed by a "flick" reading. In inhibition tests, of course, it is only necessary for one hemagglutinin to be present in an eightfold excess (in terms of titer) over the other for its activity to be measured without interference from the contaminant. In our experience, only the supernatant fluids of 5-day static cultures have commonly had an excess of F–HA over LPF–HA.

In the study of the hemagglutinins in crude preparations care should be taken that vesicular material arising from the bacterial cell membrane is absent, since this can possess both activities on the same particle and under certain circumstances give rise to anomalous results. High speed centrif-

ugation or gel-exclusion chromatography on Sepha-rose removes such material.

Relationship of LPF–HA and F–HA to the PAGE Bands of Parton and Wardlaw and to the Serotype Agglutinogens

Parton and Wardlaw (15) demonstrated that the cell walls of phase I *B. pertussis* strains could be distinguished from those of nonprotective phase IV strains by the presence in the former of proteins that gave rise to characteristic bands with molecular weights of 30,000 and 28,000 when cell walls were examined by PAGE–SDS. In Figure 5, we show by mixing purified components with cell walls that these bands are most probably distinct from those contributed to the PAGE–SDS pattern by LPF–HA, by F–HA, and by agglutinogen 2. The purification of the latter will be the subject of a separate communication (MacLennan, Hawkins and Irons, to be published). Agglutinogen 2 is also distinct from F–HA, and LPF–HA, the latter conclusion following more clearly from the separate behavior of the two substances on immunodiffusion (Fig. 6) than from the PAGE–SDS patterns.

Figure 6. Properties of antisera to LPF–HA and F–HA. Antigens: LPF is affinity chromatography purified LPF–HA, 0.25 mg/ml; F is Sepharose 6B purified F–HA, 0.15 mg/ml; Fi is Sepharose 6B purified F–HA inactive in hemagglutination, 0.13 mg/ml; Fb is crude *B. bronchiseptica* F–HA, 0.45 mg/ml; TS is phase I *B. pertussis* Tohama cell sonicate, 20 mg/ml; A2 is purified serotype 2 agglutinogen, 0.1 mg/ml. Antisera: n1, n2 and n3 are preimmunization sera to which the immune sera f1, f2, and 1pf correspond; f1 and f2 are the individual antisera and f the pooled antisera against Sepharose 6B purified F–HA; 1 pf is an antiserum against LPF–HA; a2 is a serotype Z specific antiserum prepared by adsorption of an antiserum to cells of a serotype-2 strain with cells and sonicates of a serotype 1.3 strain. Photographed after 24 hr.

Figure 5. SDS–PAGE of LPF–HA, F–HA, agglutinogen 2 and phase I and phase IV *B. pertussis* cell walls. Samples (left to right): (1) phase IV cell walls + agglutinogen 2; (2) phase I cell walls + agglutinogen 2; (3) agglutinogen 2; (4) phase IV cell walls + F–HA; (5) phase I cell walls + F–HA; (6) F–HA; (7) phase IV cell walls; (8) phase I cell walls; (9) LPF–HA; (10) phase IV cell walls + LPF–HA; (11) phase I cell walls + LPF–HA; (12) phase IV cell walls of 134 strain of *B. pertussis;* (13) phase I cell walls of 134 strain of *B. pertussis.* The arrows indicate the X-mode specific band of molecular weight 30,000 and 28,000. The mobility of these bands differs in gels 1–8 from that in gels 9–13.

Immunological Properties of LPF–HA and F–HA

Antisera were raised in pairs of rabbits by the injection of 100 μg of purified hemagglutinin in Complete Freund's Adjuvant. A single dose in a total volume of 1 ml, distributed between two intramuscular (hindquarters) and two subcutaneous (scapular region) sites was given and the rabbits were bled a month later.

Figure 6 reveals a number of features of the antibody response as demonstrated by the immunodiffusion method.

With respect to the LPF–HA antiserum:

1. The diffuse precipitation zone close to the antigen reservoir is attributable to the nonimmunological precipitation of LPF–HA by serum glycoproteins. It is given by all sera, normal or otherwise, to a varying extent.

2. The intense line of precipitation formed with LPF–HA can be shown to be given only by sonicates of phase I strains, and not by those of phase IV strains or other species of *Bordetella*. The ability to form this line can be removed by adsorption of *antigen* preparations with human erythrocyte stromata.

3. The antiserum contains antibodies that are not readily detectable with the immunizing Tohama LPF–HA preparation, in particular antibodies to agglutinogen 2 as shown here. This property may be attributed to the known adjuvant activity of LPF–HA and would make the heterogeneity of the immunological response a particularly stringent criterion of purification for this substance.

With respect to the F–HA antiserum:

1. The line illustrated here is common to active and inactive (due to storage) F–HA, and to hemagglutinin preparations from *B. bronchiseptica*. It is also present in sonicates of cells of all three species of *Bordetella,* including phase IV organisms. A second line, not illustrated, has recently been found when concentrated preparations of F–HA (>500 μg/ml) are tested. The ability to form this line is removed by adsorption of the *antigen* preparation with human erythrocyte stromata.

2. A third antigen present in the F–HA preparations can be detected with the LPF–HA antiserum.

We tentatively conclude that F–HA and LPF–HA antisera contain distinct antibodies reactive with the corresponding biologically active hemagglutinin on immunodiffusion, but that the presence of other antibody responses suggests caution in the interpretation of protection tests performed with either the antigen preparation or their antisera.

Apart from immunodiffusion, antibodies to the biologically active hemagglutinins can be selectively measured by an inhibition of hemagglutination test, as described above, provided the antiserum contains activity above the level attributable to normal serum components, i.e., sialoproteins for LPF–HA and cholesterol compounds for F–HA.

Protection Tests

These were performed in mice by a standard Kendrick assay generally using groups of 30 mice at each of three fivefold antigen dilutions, and by a novel test in rabbits. The latter was based on the observation (MacLennan, Druett, Hawkins and Broster, to be published) that rabbits and marmosets can be readily infected by exposure to *B. pertussis* aerosols (10^5 viable organisms/liter, inhaled for 5 minutes) in the apparatus of Druett and May (16).

Successful infection requires that the animals be free from active or past infection with *Bordetella bronchiseptica* as demonstrated by the results of nasal swabs and the titration of serum samples for antibodies to the lipopolysaccharide endotoxin of

B. bronchiseptica. Infection with *B. pertussis* is manifested by long-term (>50 days) colonization of the respiratory tract as revealed by nasal swabs, without obvious illness.

In the mouse test LPF–HA was toxic above a 4 μg dose and nonprotective below this level. The mean PD50 of chromatographically purified F–HA when adsorbed onto Alhydrogel was about 3 μg per mouse (8 tests). Caution must be exercised in interpreting these results because of the heterogeneity of our F–HA preparations. However, when crude F–HA is fractionated on Sepharose 6B, the pooled materials eluted from the column both ahead of and behind the active F–HA peak, as measured by hemagglutination, have poor protective activity, encouraging the view that protective activity and F–HA are closely associated.

The results of a rabbit protection test are shown in Table 7. A group of eight control animals, which

Table 7. Susceptibility of Rabbits Immunized with LPF–HA and F–HA to Respiratory Infection with *B. pertussis* Serotype 1.3 (M2 Strain)

| Immunizing Preparation | Animals infected/Animals challenged | | | | |
| | Days after challenge | | | | |
	7	14	28	35	42
Control Either Alhydrogel or Incomplete Freund's Adjuvant	8/8	8/8	8/8	8/8	6/8
Standard UK vaccine, 41 IU	4/6	4/6	4/6	3/6	3/6
LPF–HA, 100 μg Alhydrogel	2/2	2/2	2/2	2/2	2/2
LPF–HA, 100 μg Incomplete Freund's Adjuvant	2/2	2/2	2/2	2/2	0/2
F–HA, 100 μg Alhydrogel	7/7	7/7	6/7	5/7	5/7
F–HA, 100 μg Incomplete Freund's Adjuvant	3/7	5/7	5/7	4/7	4/7
Previously pertussis-infected animals, free from pertussis	0/6	0/6	0/6	0/6	0/6

All animals, in randomized groups unrelated to immunization schedule, inhaled an aerosol containing 10^5 viable organisms/l for 5 minutes on day 0. Groups had been immunized 1 month earlier with a single dose of antigen, except in the case of standard vaccine where six subcutaneous injections at intervals of 2–3 days were given, to a total dosage of ca. 41 IU/rabbit.

had received an injection of either aluminum hydroxide suspension (Alhydrogel) or Incomplete Freund's Adjuvant, were uniformly infected by the challenge dose, whereas a group of rabbits that had been previously infected with *B. pertussis* but were free from recoverable organisms at the time of rechallenge were uniformly resistant. Within this framework of susceptibility and resistance, multiple doses of a standard human vaccine (Second U.K. Standard) conferred partial immunity as did a single dose of 100 μg of F–HA in Incomplete Freund's Adjuvant. LPF–HA (100 μg) proved very toxic whether given in Incomplete Freund's Adjuvant or adsorbed onto Alhydrogel. In both circumstances 4/6 animals died within 4 days. The survivors were ill and showed massive and sustained leukocytosis.

Bordetella bronchiseptica

B. bronchiseptica strains do not appear to produce LPF–HA. When 11 strains were cultivated statically on Cohen and Wheeler medium for 3 days, the supernatants and sonicates of two strains gave hemagglutination of the F–HA character but of rather low titer. Of the 11 strains only 2 positive strains were recent animal infection isolates, being derived from natural vervet monkey infections. The hemagglutinin activity was inhibited by cholesterol liposomes but not by haptoglobin. When three groups of eight rabbits were immunized with multiple doses of whole cell vaccines prepared from F–HA inactive and active *B. bronchiseptica* strains and from a *B. pertussis* strain, only the sera of the latter two groups were inhibitory for purified *B. pertussis* F–HA, and that to an equal degree.

DISCUSSION

The LPF–HA of *B. pertussis* resembles another bacterial toxin with multiple pharmacological effects, cholera toxin, in showing specific binding to a sialo-compound receptor. The specificities do not appear to be identical, however. The binding of haptoglobin and ceruloplasmin may be of significance to the metal requirements of *B. pertussis* when growing in vivo. On the other hand, respiratory tract sialomucins may tend to dislodge bacteria adhering to cells, by binding competitively to LPF–HA. Both these metabolic and pathogenic aspects of sialoprotein binding invite investigation.

With regard to the apparently anomalous lack of effect of neuraminidase treatment of erythrocytes on agglutination by LPF–HA, in contrast to its effect on influenza virus hemagglutination, we consider that ample explanation can be found in terms of hemagglutinin-receptor valences and avidity of binding, in density of receptor sites, and in the geometry of the combination process.

The specificity of F–HA for cholesterol compounds is unique, we believe, among hemagglutinins from all sources so far described. As such it should prove valuable in studies of membrane structure and dynamics. However, it is difficult to see how a specificity for such a general cell surface component as cholesterol could provide the basis for the sort of tissue or cell-type tropism that has been invoked as a function for fimbriae in other pathogenic processes (17). With regard to protection against *B. pertussis* infection it appears that F–HA may be sufficient but not necessary, since aerobically grown cells with little or no evident F–HA can be fully protective. Moreover, it appears from recent "budget" experiments in our laboratory, that the protective activity of exhaustively extracted F–HA preparations from statically grown cultures is a very small fraction of the protectivity of the residual extracted cells. Nevertheless, F–HA may meet the requirement of an effective and nontoxic pertussis immunogen, and its economic production on a large scale then becomes important. In this connection, the apparent presence of F–HA in certain strains of *B. bronchiseptica*, an organism that is readily grown in bulk and appears to be free from LPF–HA, merits further investigation. It is noteworthy that an early study of protectivity by *B. bronchiseptica* in the Kendrick assay showed some strains to be protective (18).

ACKNOWLEDGMENT

REFERENCES

1. Arai, H.; Sato, Y. Separation and characterization of two distinct hemagglutinins contained in purified leukocytosis-promoting factor from *Bordetella pertussis*. Biochem. Biophys, Acta 444:765–782; 1976.
2. Morse, S. I.; Morse, J. H. Isolation and properties of the leukocytosis and lymphocytosis-promoting factor of *Bordetella pertussis*. J. Exp. Med. 143:1483–1502; 1976.
3. Fisher, S. The erythrocyte receptor for *Pertussis* hemagglutinin. Aust. J. Exp. Biol. Med. Sci. 27:185–189; 1949.
4. Sato, Y. Toxic factor of *Bordetella pertussis* with special reference to leukocytosis-promoting factor, histamine-sensitizing factor and hemagglutinins (In Japanese). J. Protein, Nucleic Acid and Enzyme 21:164–183; 1976.
5. Connell, G.' E.; Shaw, R. W. The purification of haptoglobin; Can. J. Biochem. 39:1013–1019; 1961.

6. Cohen, S. M.; Wheeler, M. W. Pertussis vaccine prepared with phase I cultures grown in fluid medium. Am. J. Public Health 36:371–376; 1946.
7. Smith, I., ed. Chromatographic and electrophoretic techniques, vol. 2. London: Heinemann; 1976:321–346.
8. Reisfeld, R. A.; Lewis, U. J.; Williams, D. E. Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature 195:281–283; 1962.
9. Gottschalk, A. Interaction between glycoproteins and viruses. Balazs, E. A.; Jeanloz, R., eds. The amino sugars. New York: Academic Press, Inc.; 1966:337–359.
10. Howe, C.; Lee, L. T. Virus-erythrocyte interactions. Adv. Virus Res. 17:1–50; 1972.
11. Holt, L. B. The pathology and immunology of *B. pertussis* infection. J. Med. Microbiol. 5:407–424; 1972.
12. Holmgren, J.; Lonnroth, I.; Svennerholm, L. Tissue receptor for cholera exotoxin. Infect. Immun. 8:208–214; 1973.
13. Munoz, J.; Smith, R. F.; Cole, R. L. Some physical properties of histamine-sensitizing factor. Symp. Series Immunobiol. Stand. 13:265–270; 1969.
14. Lehrer, S. B.; Tan, E. M.; Vaughan, J. H. Extraction and partial purification of the histamine-sensitizing factor of *Bordetella pertussis*. J. Immunol. 113:18–26; 1974.
15. Parton, R.; Wardlaw, A. C. Cell envelope proteins of *Bordetella pertussis*. J. Med. Microbiol. 8:47–57; 1974.
16. Druett, H. A.; May, K. R. A wind tunnel for the study of airborne infections. J. Hyg. (Camb.) 50:69–81; 1952.
17. Nagy, B.; Moon, H. W.; Isaacson, R. E. Colonisation of porcine intestine by enterotoxigenic *E. coli*. Infect. Immun. 16:344–352; 1977.
18. Kendrick, P. L.; Nadolski, E. B.; Eldering, G.; Baker, J. Antigenic relationships of *H. pertussis,* the parapertussis bacillus and *B. bronchiseptica* as shown by cross protection tests in mice. J. Bacteriol. 66:166–169; 1953.

Toxicity and Potency of a Purified Pertussis Vaccine

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ABSTRACT

The preparation, properties, toxicities and immunogenicity of a partially purified pertussis vaccine are described. The vaccine was prepared from the surface components of phase I *Bordetella pertussis* by removing toxic components as much as possible by means of Sepharose 4B column chromatography and sucrose density gradient centrifugation. The vaccine contained 5–10 μg protein-derived nitrogen per ml, had a hemagglutination titer of 1/512 before inactivation and addition of aluminum phosphate gel, and provided significant protection and K-agglutinin production in mice. The ED_{50} was 0.11–0.39 μg protein-N against intracerebral challenge infection. There was only a trace of histamine sensitizing activity in the vaccine and very little lymphocytosis promoting activity since it produced only a 1.4- to 2.0-fold increase of leukocyte population in mice. The endotoxic activity was less than 1% of the value of whole-cell vaccine by pyrogen and limulus tests. In a preliminary clinical trial, the vaccine did not show any significant reaction in human infants.

INTRODUCTION

Many efforts have been made to separate the protective antigen (PA) from toxic components of *Bordetella pertussis* (1–4). We have reported that purified preparations of biologically active components of the organisms such as heat-labile dermonecrotic toxin (HLT), O-agglutinogen [endotoxin (ET)], K-agglutinogen (KA), histamine sensitizing factor (HSF), and hemagglutinating substance (HA), were not effective in protecting mice against intracerebral challenge as shown in Table 1 (5–9). However, a mixture of the HA and a small amount of leukocytosis promoting factor (LPF) provided significant protection in mice without giving any notable toxicity (Table 2) (10). These results led us to attempt to purify the immunogenic fraction and remove most of the toxic components of phase I organisms.

MATERIALS AND METHODS

B. pertussis phase I strain Maeno possessing agglutinogens 1, 2, and 3 was used.

Whole Cell Pertussis Vaccine

For mouse protection testing, Japanese standard pertussis vaccine Lot #28, which contained 14.4 IPU per 20 IOU, was used. It was a gift from Dr. Sato of the Japanese National Institute of Health. For toxicity testing, a diphtheria-tetanus-pertussis combined vaccine, Lot #761, a gift from Dr. Kurokawa of the National Institute, was used as a reference. Whole cell vaccine, prepared from strain Maeno according to the Japanese Minimum Requirements of Biologic Products (11), was also used as a reference.

Animals

Female ddN mice, 3 to 4 weeks old, were used for potency and toxicity tests. The same kind of mice 5 to 6 weeks old were used for LPF and HSF tests.

Biological Assays

HA activity was determined using chicken red cells as described by Sato and Arai (12). A minimal quantity producing complete hemagglutination was read.

LPF was examined by the method of Morse and Bray (13), using a Model D Coulter Counter (Coulter Electronics Inc., Hialeah, Florida) for counting leukocytes. One unit of LPF activity was arbitrarily defined as the amount producing a twofold increase in total leukocyte population as compared with that of the untreated mice.

HSF was assayed by the method of Parker and Morse (14). The mice were challenged intraperitoneally with histamine dihydrochloride (Wako Pure Chemical Industries Ltd., Osaka, Japan) in phosphate-buffered saline, pH 7.0, containing 2.4 mg histamine base in a 0.5 ml dose.

ET was examined by four different methods: the mouse body-weight decreasing test by the method

Table 1. Mouse Protective Activity of Purified Components of *Bordetella pertussis*

Purified Preparations	Biological Activity						Mouse Protective Activity (ED$_{50}$) µg
	HLT (MND)[a] µg	KA[b] µg	ET[c] µg/ml	HSF (HSD$_{50}$)[d] µg	HA (MHD)[e] µg/ml	LPF[f] µg	
Heat-labile toxin	0.01	—[g]		—	—	—	—
O-Agglutinogen (Endotoxin)	—	—	0.0001	—	—	—	—
K-Agglutinogen	—	15		—	—	—	—
Histamine-sensitizing factor	—	—	>0.05	0.01	—	>10	—
Hemagglutinating substance	—	—	>0.1	—	0.08	—	>27.7
Leukocytosis-promoting factor	—	—	>0.1	3.0	—	0.6	—
Whole cells	0.6	250	2.43	20.1	4.0	24.4	4.9

[a] MND, a minimum quantity producing complete dermatonecrotic lesion
[b] a minimum quantity adsorbing agglutinin of 1:2
[c] a minimum quantity producing coagulation in Pre-gel test
[d] HSD$_{50}$, dose sensitizing 50% of the mice to the lethal effect of histamine
[e] MHD, a minimum quantity producing complete hemagglutination
[f] Dose causing twofold increase in leukocyte population
[g] Dash means not detected in 50 µg amounts

Table 2. Mouse Protective Activity of Purified HA and LPF, and a Mixture of HA and LPF

Purified HA (µg/mouse)	Purified LPF (µg/mouse)	Alum-gel (µg/mouse)	Mouse Protection Test Survivals/ Tested	ED$_{50}$ (HA µg)
1	—	—	2/10	
5	—	—	4/10	>27.7
25	—	—	4/10	
1	—	100	2/10	
5	—	100	5/10	>14.7
25	—	100	5/10	
1	0.4	—	1/10	
5	0.4	—	7/10	3.6
25	0.4	—	9/10	
1	0.4	12.5	6/10	
5	0.4	12.5	8/10	0.8
25	0.4	12.5	10/10	
	2.0	—	3/10	
5	2.0	—	7/10	2.5
25	2.0	—	8/10	
1	2.0	12.5	5/10	
5	2.0	12.5	10/10	<0.8
25	2.0	12.5	10/10	
—	2.0	12.5	2/10	
—	2.0	25.0	2/10	—
—	2.0	100.0	1/10	
—	—	—	0/10	—

of Kurokawa et al. (15); change in peripheral leukocyte population by the method of Kurokawa et al. (16); a pyrogen test according to the Japanese Minimum Requirements of Biologic Products (11); and a limulus coagulation test using both Pyrostat (Worthington Biochemical Corporation, N.J.) and Pre-gel (Teikoku Hormon Mfg. Co. Ltd., Tokyo, Japan) according to the manufacturers' instructions.

HLT was tested in guinea pig as described previously (5).

Both mouse protection and safety tests were performed according to the Japanese Minimum Requirements of Biologic Products (11).

The test for the presence of agglutinogens in fractions was performed in mice by the method of Munoz and Bergman (4).

Chemical Assays

The nitrogen derived from protein was determined by the Kjeldahl method, and sugar by the anthrone method.

RESULTS

Extraction and Partial Purification of PA

Figure 1 outlines the purification procedure.

Extraction of surface components. The organisms grown at 36°C for 2 days on the solid Cohen-Wheeler-Powell medium were harvested in 0.002
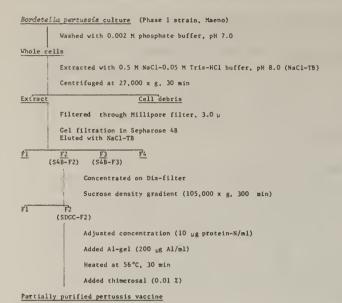
Figure 1. Outline of purification procedure.

Figure 2. **Sepharose 4B column chromatograph of a 1 M NaCl–TB extract.** 100 ml of the extract was sieved through Sepharose 4B in a 4.0 × 86 cm column. Elution was at a flow rate of about 40 ml/hr with NaCl–TB. 10–12 ml fractions were collected. Fractions 78–110 were pooled. The figure shows absorbance at 280 nm (•——•) and hemagglutinating activity (x····x).

M phosphate buffer, pH 7.0, and washed with the buffer by centrifugation at 27,000 × g at 4°C for 30 minutes. The organisms, which agglutinated chicken red cells in a concentration of less than 2.4 × 10^8 organisms/ml, were suspended to a density of 5 × 10^{11} in 0.05 H Tris buffer containing 0.5 M NaCl, pH 8.0 (NaCl–TB). After centrifugation at 27,000 × g for 30 minutes, the supernatant fluid was collected and filtered through a Millipore filter, 3.0 μ pore diameter (Millipore Filter Co., Bedford, Mass.).

Removal of LPF and HSF from the extract. To remove most of the LPF and HSF, the filtrate was passed through a Sepharose 4B (Pharmacia Fine Chemical Inc., Piscataway, N.J.) column equilibrated with NaCl–TB. By eluting with the buffer, the second protein peak (S4B–F2), which contained a potent HA in mean titer of 1/512, was collected (Fig. 2). The HA-rich fraction was slightly contaminated by LPF and HSF derived from tailing of the third protein peak (S4B–F3), which was rich in LPF and HSF. Without completely removing the contaminating LPF, the HA-rich fraction was concentrated on a Diafilter apparatus using a G–10T membrane (Bio-Engineering Co. Ltd., Tokyo, Japan).

Removal of ET from the HA-rich fraction. To remove ET, the HA-rich fraction was layered on a sucrose density gradient and centrifuged in an RPS–25 rotor with a Hitachi preparative ultracentrifuge, Model 55P–2 (Hitachi, Ltd., Ibaragi, Japan) at 105,000 × g for 300 minutes. Most HA

activity was recovered in the fraction from 8 to 10% sucrose density (SDGC–F2), as shown in Figure 3. ET activity recovered in the fraction with sucrose density over 13% (SDGC–F1) was discarded.
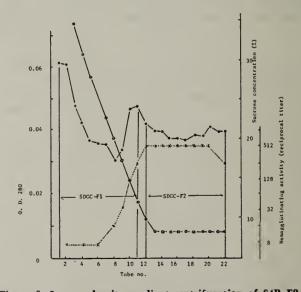
Figure 3. **Sucrose density gradient centrifugation of S4B–F2.** S4B–F2 was layered on a sucrose gradient, which consisted of a 25-ml portion of 10–30% sucrose in 0.015 M citric acid, pH 7.0, and 3-ml portion of 60% sucrose in the same solution as a bed, for 30 ml portion of the fraction. The tubes were centrifuged at 105,000 × g for 300 min. The figure shows absorbance at 280 nm (•——•), sucrose concentration (○——○), and HA activity (x-----x). Fractions 12–22 pooled.

Preparation of the partially purified vaccine. The HA-rich SDGC–F2 fraction was adjusted to a protein-nitrogen concentration of 5–10 μg N/ml to give an HA titer of 1/512. Aluminum phosphate gel was added to the fraction slowly with constant stirring until a concentration of 200 μg Al/ml was reached. To inactivate trace HLT, the preparation was heated at 56°C for 30 minutes. Finally, thimerosal was added to a final concentration of 0.01%.

Recovery of biological activities in the purification steps. Table 3 shows typical recoveries of PA, HA, LPF, and HSF activities during the purification procedure. Most cellular HA was extracted in NaCl–TB, with about 15% of PA in the cells. About 50% of the HA was recovered in SDGC–F2 with a 32-fold increase in specific activity. Most PA in the NaCl–TB extract was recovered in SDGC–F2 with about 7.8-fold increase in specific activity. Most LPF with HSF was removed from S4B–F2 by gel filtration. No parallelism was observed among PA, HA, and LPF activities and recoveries in the fractions obtained in the purification process.

As shown in Figure 4, the Pyrostat test indicated that about 5% of ET activity in the starting material was found in the NaCl–TB extract; over 80% of the activity was removed by Sepharose 4B gel-

filtration and sucrose density gradient centrifugation.

General Properties of SDGC–F2

The SDGC–F2 had significant hemagglutinating activity at a dilution of 1/512 per 10 μg N/ml, but lost it when heated at 56°C. The preparation precipitated optimally at pH 4.6 or by dialysis against water. It was most soluble at pH 8–10, at high salt concentration (0.4 to 1.0 M NaCl), or in 4 M urea and insoluble in distilled water or in low salt concentration. It contained 14.8% protein-derived nitrogen and 3.6% sugar. Electron microscopy of the preparation revealed the presence of filamentous molecules measuring about 2×40 nm and a small amount of spherical molecules about 10 nm in diameter.

Immunogenicity and Toxicity of the Purified Vaccine in Laboratory Animals

Mouse protective potency and LPF, HSF, and ET toxicities of the purified vaccine were compared with those of the whole cell vaccines. In the mouse potency test, there was no significant difference between the purified vaccine and the standard vaccine (Table 4). The ED_{50} of the purified vaccine was 0.11 to 0.39 μg N against intracerebral chal-

Table 3. Recovery of PA, HA, LPF, and HSF Activities in the Purification Steps

				Purification Step		
		Starting Material	NaCl–TB Extract	Sepharose 4B		SDGC F2
				S4B–F2	F4B–F3	
Volume (ml)		1,000	1,000	2,000	1,000	2,000
Protein–N (μg/ml)		1,300	56	12	13	10
PA	ED_{50} (dil.)	833	125	50		50
	ED_{50}(dil.) (μg prot–N/ml)	1.56	0.45	0.24		0.20
	Sp act (per μg prot–N)	0.64	2.23	4.17		5.00
	Purification (fold)	1.0	3.5	6.5		7.8
	Recovery (%)	100	15.0	12.0		12.0
HA	MHD [a] (dil)	2,084	2,084	512	32	512
	(μg prot–N/ml)	0.67	0.03	0.02	0.41	0.02
	Sp act (per μg prot–N)	1.58	36.57	42.67	2.46	51.20
	Purification (fold)	1.0	23.1	27.0	1.5	32.4
	Recovery (%)	100	100	50.0	1.5	50.0
LPF	LPU/ml		27.0	1.3	21.6	1.0
	Sp act (per μg prot–N)		0.48	0.11	1.66	0.10
	Purification (fold)		1.0	0.23	3.46	0.21
	Recovery (%)		100	9.6	80.0	7.4
HSF	HSD_{50} (dil)		77.1	5.6	38.6	1.0
	Sp act (per μg prot–N)		1.37	0.47	2.97	0.10
	Purification (fold)		1.0	0.3	2.2	0.1
	Recovery (%)		100	14.5	50.1	2.6

[a] Minimum quantity producing complete hemagglutination.
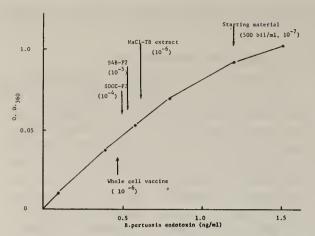
Figure 4. Limulus test of the preparations in purification steps. Purified *B. pertussis* endotoxin was used for the standard curve by Pyrostat. ( ) shows the dilution of test sample.

lenge. The purified vaccine as well as the whole cell vaccine produced an agglutinin of 1:160 to 1:320 against the agglutinogen prepared from phase I organisms. The protective and agglutinin-producing activities of the vaccine were stable for a week at 37°C and for at least a year at 4°C.

The purified vaccine had no detectable HSF activity in 5 μg N amounts, and in mice had only slight LPF activity that increased leukocyte population 1.4- to 2-fold (Table 5).

In assays for ET, the whole-cell vaccine caused a fall in mouse body-weight of about 1.0 to 1.5 g 24 hours after inoculation, while the purified vaccine caused a fall of less than 0.1 g. The whole cell vaccine reduced 32–40% of the leukocyte population within 2 hours after inoculation, but the purified vaccine did not affect the total leukocyte count (Table 5). The whole cell vaccine was pyrogenic in rabbits in a dilution of 1:500, but the purified vaccine was not pyrogenic in a dilution of 1:50 (Fig. 5). The results of limulus tests indicated that the ET activity in the purified vaccine was less than 1% of that in the whole-cell vaccine.

When given intracutaneously in guinea pigs, the purified vaccine did not produce any visible local reaction. No abnormal toxicities were observed in guinea pigs or mice inoculated wth the purified vaccine.

Clinical Trial of the Purified Vaccine

The purified vaccine, Lot #45, was preliminarily tested in infants 1.2 to 3.1 years of age by the members of the Improved Pertussis Vaccine Committee (Dr. Fukumi). It did not give any adverse reactions, but it produced agglutinin against phase I agglutinogen, with a titer of 1:160 to 1:320 about 1 month after a second injection.

Table 4. Mouse Protective Activity of Purified Vaccine

Vaccine No.	ED$_{50}$		1 S.D.		C.R.[a]	IPU/ml
	log(dil.)	Protein–N	log(dil.)	%		
45	0.9855	0.15	0.0517	56–178	−0.4	18.9
Standard [b]	0.8667	0.78	0.0679	55–181		14.4
52	1.3979	0.14	0.2542	56–180	−0.5	20.8
53	1.4888	0.11	0.2122	52–163	−0.9	25.5
Standard	1.2371	0.78	0.1768	58–150		14.4
57	1.3280	0.28	0.1282	76–163	0.7	10.5
Standard	1.4678	0.78	0.1282	74–135		14.4
58	1.3209	0.34	0.1525	70–142	1.0	8.3
Standard	1.5587	0.78	0.1768	67–150		14.4
60	1.3001	0.39	0.1433	72–139	1.5	7.3
Standard	1.5936	0.78	0.1415	62–161		14.4
61	1.0833	0.34	0.1083	78–128	1.5	8.6
Standard	1.3071	0.78	0.1066	78–128		14.4

[a] C.R. = Critical Ratio.

$$CR = \frac{\text{log(dil) ED}_{50}\text{ Standard Vaccine} - \text{log(dil) ED}_{50}\text{ Test Vaccine}}{\sqrt{[\text{log(dil) 1 S.D. Standard Vaccine}]^2 + [\text{log(dil) 1 SD Test Vaccine}]^2}}$$

[b] Standard pertussis vaccine, Lot # 28.

Table 5. LPF, HSF and ET Activities of Purified Vaccine

Vaccine No.	LPF (unit)	HSF			HSD$_{50}$	ET Weight (g) [a] gain/mouse	Leukocyte [b] decrease (2hr)
		dil. 5^0	5^{-1}	5^{-2}			
45	0.8	2/5	0/5	0/5 [c]	<1.0	−0.1	0
52	0.4	0/5	0/5	0/5		+0.1	0.03
53	0.7	0/5	0/5	0/5		0	0.02
57	0.5	1/5	0/5	0/5	<1.0	0	0
58	1.0	0/5	0/5	0/5		0	0
61	1.0	1/5	0/5	0/5	<1.0	0	0
Whole cell vaccine (71)	3.0	4/5	3/5	0/5	4.2	−1.0	32
Reference vaccine (761)	2.1	5/5	3/5	0/5	5.9	−1.5	40
Standard vaccine (28)	1.7	5/5	3/5	0/5	5.9	−0.4	32

[a] Mouse body-weight gain 24 hr after inoculation.
[b] The decreasing rate of total leukocyte population:

$$\frac{\text{mean number before injection} - \text{mean number 2 hr after injection}}{\text{mean number before injection}} \times 100$$

[c] Deaths/tested.
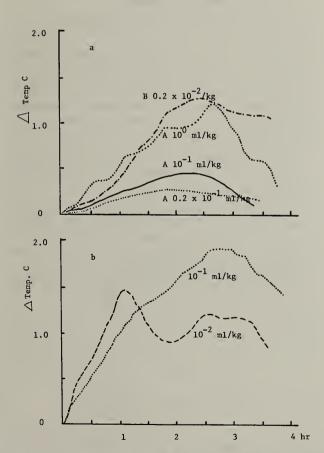
Figure 5. Typical fever curves of the preparations, using a Pyrogen test recorder, model EP 76–12 (Iio Electron. Co., Ltd., Tokyo, Japan). a. (A) partially purified vaccine, and (B) whole-cell vaccine. b. partially purified vaccine prepared from the residue removed HA.

DISCUSSION

Many attempts have been made to prepare a purified PA for use as a nontoxic pertussis vaccine (2,4). Recently, Sato indicated that purified HA separated from HSF was a protective antigen (17). In our previous work, a single HA which had been repeatedly purified, as well as a single purified preparation of other biologically active components such as HLT, KA, ET, and HSF, did not give mice any significant protection against intracerebral challenge (5–9). On the other hand, a mixture of the HA and a low content of LPF, neither of which had protective activity alone, induced a significant protection in mice (10). These results and some conflicting results as to the exact nature of PA moeity (4), suggest either that PA may be induced by an association of both HA and LPF activities, or that some other unknown factor that might be found in both HA and LPF fractions may confer protection. In light of our previous findings, we attempted to prepare a partially purified vaccine from an extract of surface components of phase I organisms by removing toxic components as much as possible, especially ET, HSF, and LPF. The vaccine prepared from the HA-rich fraction containing minimum LPF activity gave significant protection to mice without causing significant toxicity. In the purification process, HA also was helpful as an indicator for identification of a PA-

rich fraction. After the HA was extracted, the high PA activity still remained in the cell residue, which lost hemagglutinating activity. Although the PA could be released by sonic treatment, it was difficult to remove the most toxic components, especially ET, from the sonic extract, probably because of particle-size polydispersity of the ET.

Several questions remain unanswered regarding the antigens participating in the induction of immunity protecting mice from intracerebral infection with *B. pertussis*.

In preliminary tests the purified vaccine showed no significant adverse reaction in infants, but further clinical trials are needed to evaluate its applicability.

ACKNOWLEDGMENTS

REFERENCES

1. Pittman, M. *Bordetella pertussis*—bacterial and host factors in the pathogenesis and prevention of whooping cough. Mudd, S., ed., Infectious agents and host reactions. Philadelphia: Saunders; 1970:239–270.
2. Munoz, J. Protein toxins from *Bordetella pertussis*. Kadis, S.; Montie, T. C.; Ajl, S. J., eds. Microbial toxins. New York and London: Academic Press, Inc.; 1971:271–300.
3. Morse, S. I. Biologically active components and properties of *Bordetella pertussis*. Perlman, D., ed. Advances in applied microbiology. New York, San Francisco, and London: Academic Press, Inc.; 1976:9–26.
4. Munoz, J. J.; Bergman, R. K. The *Bordetella pertussis* cell and its biological activities in animals. *Bordetella pertussis*. Immunological and other biological activities. New York: Marcel Dekker, Inc.; 1977:13–70, 195–208.
5. Nakase, Y.; Takatsu, K.; Tateishi, M.; Sekiya, K.; Kasuga, T. Heat-labile toxin of *Bordetella pertussis* purified by preparative acrylamide gel electrophoresis. Jpn. J. Microbiol. 14:359–366; 1969.
6. Nakase, Y.; Tateishi, M.; Sekiya, K.; Kasuga, T. Chemical and biological properties of the purified O antigen of *Bordetella pertussis*. Jpn. J. Microbiol. 14:1–8; 1970.
7. Nakase, Y.; Kasuga, T. Purified K-agglutinogen of *Bordetella pertussis* and its properties. Jpn. J. Microbiol. 15:247–256; 1971.
8. Doi, M.; Nakase, Y.; Kasuga, T. Separation and purification of histamine-sensitizing factor in *Bordetella pertussis*. Jpn. J. Bacteriol. 27:70; 1972. (In Japanese.)
9. Nakase, Y.; Doi, M.; Kasuga, T. Hemagglutinating substance of *Bordetella pertussis*. Jpn. J. Med. Sci. Biol. 28:57–58; 1975.
10. Doi, M.; Asahara, T.; Takatsu, K.; Nakase, Y. Mouse protective activities of hemagglutinating substance and K-agglutinogen of *Bordetella pertussis*. Jpn. J. Bacteriol. 33:70; 1978. (In Japanese.)
11. Ministry of Health and Welfare, Japanese Government. Minimum requirements of biologic products; pertussis vaccine. 1973:165–176. (In Japanese.)
12. Sato, Y.; Arai, H. Leukocytosis-promoting factor of *Bordetella pertussis*. II. Biological properties. Infect. Immun. 7:992–999; 1973.
13. Morse, S. I.; Bray, K. K. The occurrence and properties of leukocytosis and lymphocytosis-stimulating material in the supernatant fluids of *Bordetella pertussis* cultures. J. Exp. Med. 129:523–550; 1969.
14. Parker, C. W.; Morse, S. I. The effect of *Bordetella pertussis* on lymphocyte cyclic AMP metabolism. J. Exp. Med. 137:1078–1090; 1973.
15. Kurokawa, M.; Ishida, S.; Asakawa, S.; Kuratsuka, K. Attempts at analysis of toxicity of pertussis vaccine. I. Body weight-decreasing toxicity in mice. Jpn. J. Med. Sci. Biol. 21:137–153; 1968.
16. Kurokawa, M.; Ishida, S.; Goto, N.; Kuratsuka, K. A new method for biological assay of endotoxin using change in peripheral leukocyte population in mice as a response. Jpn. J. Med. Sci. Biol. 27:173–189; 1974.
17. Sato, Y. Toxic factors of *Bordetella pertussis*, with special reference to leukocytosis-promoting factor, histamine-sensitizing factor and hemagglutinins. (In Japanese) J. Protein, Nucleic Acid and Enzyme 21:164–183; 1976.

Suppressive Mechanisms of in vitro Antibody Response to Sheep Erythrocytes by the Lymphocytosis Promoting Factor (LPF) of *Bordetella pertussis*

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ABSTRACT

Lymphocytosis promoting factor (LPF) can selectively stimulate DNA synthesis in murine T-cells and cortisone-resistant thymocytes. Mitogenic concentrations of LPF, when added to spleen cell cultures, suppress primary and secondary plaque-forming cell (PFC) responses to sheep red blood cells (SRBC). The action of LPF is most effective during the inductive phase of in vitro antibody response. The suppression of antibody response by LPF is due to LPF-activated suppressor cells and/or their products in culture supernates. A suppressor factor, which is produced in the absence of SRBC, can nonspecifically suppress primary and secondary anti-SRBC PFC responses. The suppressive activity of this factor was removed completely by passage through an immunoadsorbent column in which anti-thymocyte serum is coupled to Sepharose 4B. This fact indicates that the suppressor factor possesses the same antigenicity as the lymphocyte antigen on the membrane of murine thymocytes.

INTRODUCTION

Lymphocytosis-promoting factor (LPF) was shown to be a T-cell mitogen, but its spectrum of activity on murine thymocyte populations differed from that of concanavalin A (Con A) (1,2). We demonstrated that mitogenic concentrations of LPF suppressed the primary plaque-forming cell (PFC) response to sheep red blood cells (SRBC) in spleen cell cultures from normal C57BL/6 mice (3). The suppressive effect of LPF could be also observed on the PFC response in the cultures using spleen cells of mice 22 to 23 weeks after adult thymectomy, whereas the suppression induced by Con A was abrogated completely when the spleen cells of adult-thymectomized mice were used (3). These facts suggest that the LPF-induced suppression of in vitro antibody response is not mediated by the short-lived suppressor cells but depends on different mechanisms. In the present study the suppression of in vitro PFC response by LPF was shown to be mediated by suppressor cells activated by LPF and/or a suppressor factor(s) produced by these cells and possessing no antigen specificity.

MATERIALS AND METHODS

Preparation of LPF

Culture fluid of *Bordetella pertussis* strain Maeno (phase I) was precipitated by 90% $(NH_4)_2SO_4$ saturation. The precipitates were washed with saline, solubilized with 0.1 M Tris-NaCl (pH 10) (Tris-NaCl buffer) and filtered through a Sephacryl S–200 column ($\phi 2.5 \times 90$ cm, Pharmacia Fine Chemicals, Sweden) equilibrated with the Tris-NaCl buffer. Fractions possessing the LPF activity were pooled and rechromatographed with the same column to obtain F–1 and F–2 fractions with LPF activity. The F–1 fraction showed a single spur in an immunodiffusion analysis using anti-*Bordetella pertussis* vaccine horse serum, whereas the F–2 fraction contained other component(s) in addition to that present in the F–1 fraction. Therefore, the F–1 fraction was used as LPF in the present study. An intravenous injection of 2 μg of the F–1 fraction resulted in 4.5-fold rise in the peripheral leukocyte count, from $12.6 \times 10^3/$mm^3 to $65.6 \times 10^3/$mm^3.

The mitogenicity of the F–1 fraction, along with Con A (twice crystallized, Miles Laboratories Inc., U.S.A.) and lipopolysaccharide (LPS) from *Salmonella typhimurium* LT$_2$, was tested by tritiated thymidine uptakes of cultured cells as described previously (3). The F–1 fraction had T-cell mitogenicity since it and Con A stimulated DNA synthesis of spleen cells from nu/+ mice but did not stimulate DNA synthesis of nu/nu spleen cells, which have a very low T-cell population. Normal thymocytes of BALB/c mice 8 weeks of age (Shizuoka Agricultural Corporation for Laboratory Animals, Shizuoka) were stimulated markedly by

2 μg of Con A but very weakly by 10 μg of the F–1 fraction. Cortisone-resistant thymocytes responded well to both the F–1 fraction and Con A. These results coincide with those reported by Kong and Morse (1,2).

RESULTS

Effect of LPF on in vitro Primary and Secondary Anti-SRBC PFC Responses

The antibody response of in vitro cultured spleen cells to SRBC was performed using the Marbrook bottle as described previously (3). Briefly, 1 ml of a cell suspension containing 2 × 10⁷ cells and 5 × 10⁶ SRBC was placed in an inner glass tube covered at the bottom with a dialysis membrane. The tube was placed in a bottle containing 10 ml of RPMI 1640 medium (Grand Island Biological Co., U.S.A.) supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Co., U.S.A., Lot no. A863019), 5 mM HEPES (Sigma Chemical Co., U.S.A.), 100 μM 2-mercaptoethanol (Wako Chemical Ind., Tokyo) and antibiotics (which were used for all cell cultures), and incubated at 37°C for 4 days under a continuous flow of 5% CO_2:95% air mixture. The number of anti-SRBC IgM PFC per culture was determined by Cunningham's hemolytic plaque chamber (4). As shown in Table 1, 10 μg of LPF could suppress IgM PFC response specific to SRBC in cultures of spleen cells from normal C57BL/6 mice or those primed with 1 × 10⁶ or 1 × 10⁸ SRBC 10 weeks earlier, indicating that LPF suppresses both primary and secondary IgM PFC responses. Figure 1 shows that LPF-induced suppression of antibody response in vitro was marked when LPF was added at the initiation of cultivation but not when added later. This indicates that the action of LPF is most effective during the inductive phase of in vitro antibody response, a property similar to that of Con A described by Rich and Pierce (5).

Suppressor Cells and Suppressor Factor Induced by LPF

To determine whether the suppression of antibody response induced by LPF is due to direct cytotoxic action on lymphocytes or to generation of suppressor cells, the following experiments were carried out: 2 × 10⁷ spleen cells, together with 10 μg of LPF, were incubated in the Marbrook bottle in the same manner as for the antibody response testing. Control cultures contained phosphate-buffered saline instead of LPF. After 2 days of incubation spleen cells were pooled and washed three times with the culture medium to remove LPF present on the cell surface. The addition of LPF-activated and washed cells to normal cell cultures significantly decreased the IgM PFC response (Table 2). However, the possibility still remains that this suppression of PFC response might be due to LPF carryover on the activated cells. We then attempted to find competitive inhibitors of the mitogenicity of LPF. The degree of inhibition or stimulation of various sugars was determined by the addition of a final concentration of 0.1 M to cell cultures containing 10 μg of LPF. Each column in Figure 2 was obtained by dividing the uptake cpm of ³H-thymidine in cultures with test sugar by that in control cultures without the sugar and multiplying by 100. D-mannose is obviously the most effective inhibitor. Rodriguez et al. (6) reported that more than 96% of Con A bound to the cell membrane could be removed by treatment with 0.1 M α-methyl-D-mannoside (α-MM), a competitive

Table 1. Suppressive Effect of LPF on the in vitro Primary and Secondary Antibody Response to SRBC

Source of Spleen Cells [a]	Mitogens	Day 4—PFC/Culture [b]	Suppression
Normal animals	Nil	788 ± 260	
Normal animals	LPF(10 μg)	242 ± 37	30.7% (p<0.005)
10⁶ SRBC primed	Nil	2,794 ± 906	
10⁶ SRBC primed	LPF(10 μg)	471 ± 62	16.9% (p<0.001)
10⁸ SRBC primed	Nil	1,589 ± 410	
10⁸ SRBC primed	LPF(10 μg)	240 ± 101	15.18% (p<0.001)

[a] C57BL/6 mice from Shizuoka Agricultural Corporation for Laboratory Animals, 8 to 18 weeks of age, were killed by cervical dislocation, and spleens were removed aseptically and teased gently in the cold medium. The cell suspension was passed through a 200-gauge stainless steel sieve, centrifuged (600 × g for 10 min), and resuspended. Only preparations with greater than 90% cell viability were used.

[b] Arithmetic means ± SD of pentaplicate cultures

Figure 1. Effect of LPF added to SRBC-stimulated spleen cell cultures at various times after culture initiation. The horizontal dashed line represents the PFC responses in control culture. Arithmetic means of pentaplicate cultures.

is not due to direct cytotoxic action of LPF but to suppressor cells activated by LPF.

We then attempted to determine whether a suppressor factor(s), produced by the LPF-activated suppressor cells, is released into culture supernates. To obtain the suppressor factor and control factor, 10 μg of LPF was added at initiation or termination of cultures. Spleen cells (2×10^7) were incubated for 2 days in the Marbrook bottle under the conditions used for antibody formation in vitro. After harvesting, free LPF in the culture supernates was removed by passage through an immunoadsorbent column coated with an amount of anti-*Bordetella pertussis* vaccine horse serum large enough to adsorb the amount of LPF used. LPF-free culture supernates were assayed in a final concentration of 1:10 to 1:20. Addition of these factors to the cell cultures was done at the start of cultivation. Although the control factor, produced in culture supernatants to which LPF was added at culture termination, possessed weak suppressive activity due to spontaneous release of suppressor factor, the addition of the suppressor factor to cell cultures led to marked nonspecific suppression of the anti-SRBC PFC response of spleen cells from normal or SRBC-primed mice (Table 3). This activity of suppressor factor was removed completely by passage through an immunoadsorbent column coupled with anti-thymocyte serum (ATS), prepared as described previously (7), to Sepharose 4B (Pharmacia Fine Chemicals, Sweden) as shown in Table 4. This fact shows that the suppressor factor possesses a surface marker of thymocytes on its molecule. Therefore the suppressor factor seems to be produced by T-cells possessing suppressive activity. Studies are now in progress to learn whether the suppressor factor can act across the murine H–2 barrier, and on the cell types (T-cells, macrophages or others), that respond to the suppressor factor.

inhibitor of Con A binding. By the method of Rodriguez et al. (6), LPF-activated cells were incubated with 0.1 M D-mannose at 37°C for 1 hour and washed twice with 0.1 M D-mannose. The washed LPF-activated cells still suppressed nonspecifically the PFC response to SRBC (Table 2). Even if a minimal amount of LPF were carried over on the surface of cultured cells, its suppressive effect to normal cells would be negligible, since the addition of 1 μg of LPF to spleen cell cultures augments rather than inhibits the in vitro antibody response to SRBC (3). These facts seem to favor the view that the suppression of PFC response by LPF

Table 2. Suppression of PFC Response by Cultured Spleen Cells Activated by LPF

Activated With	Cells	Washed with	Normal[a] cells	Day 4–PFC/Culture [b]	p Value
PBS	1×10^7	Medium	2×10^7	559 ± 95	
LPF [c]	1×10^7	Medium	2×10^7	$229 \pm 53 \downarrow$	p<0.001
PBS	1×10^6	0.2 M Man	2×10^7	$1,252 \pm 142$	
LPF	1×10^6	0.01 M Man	2×10^7	$876 \pm 148 \downarrow$	p<0.001

[a] Normal spleen cells of BALB/c mice 8 weeks of age were used.
[b] Arithmetic means ± SD of pentaplicate cultures
[c] 10 μg of LPF was used.
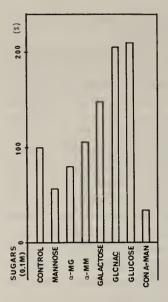
Figure 2. Screening for the inhibitor of LPF mitogenicity. α–MG: α–methyl-D–glucoside, α–MM: α–methyl-D–mannoside and GLUNAC: N–acetyl–D–glucosamine. CON A–MAN: 2 μg of Con A and 0.1 M mannose.

Table 3. Suppressive Effect of Culture Supernates on the in vitro Primary and Secondary Antibody Response to SRBC

Supernates	Spleen Cells from	Day 4–PFC/Culture [b]	Suppression
Nil (medium)		907 ± 107	
Control factor	Normal BALB/c	532 ± 97]*	59%
Suppressive factor		231 ± 106]*	25
Nil (medium)		3,599 ± 909	
Control factor	SRBC-primed BALB/c [b]	2,633 ± 597]**	73
Suppressor factor		1,348 ± 928]**	38

[a] Arithmetic means ± SD of pentaplicate cultures. *p<0.01 and **p<0.05 (expressed in comparison with each control response).

[b] Mice were primed with 5 × 10⁵ SRBC 4 weeks earlier.

Table 4. Adsorption of Suppressor Factor with Anti-Thymocyte Serum Coupled to Sepharose 4B

Supernates	Adsorbed with	Day 4–PFC/Culture [a]
Nil (medium)	—	729 ± 154]*
Control factor	—	288 ± 102]*
Suppressor factor	—	49 ± 32
Suppressor factor	ATS [b]	697 ± 120

[a] Arithmetic means ± SD of pentaplicate cultures. *p<0.001 (expressed in comparison with the PFC number of control factor group).

[b] Anti-thymocyte serum (ATS) at a dilution of 1:16 could have killed 100% of the thymocytes, 40% of the spleen cells and less than 5% of the bone-marrow cells. The dilution titer of this anti-serum to achieve 50% cytotoxicity was 1:512.

REFERENCES

1. Kong, A. S.; Morse, S. I. The in vitro effect of *Bordetella pertussis* lymphocytosis-promoting factor on murine lymphocytes. I. Proliferative response. J. Exp. Med. 145: 151–162; 1977.
2. Kong, A. S.; Morse, S. I. The in vitro effect of *Bordetella pertussis* lymphocytosis-promoting factor on murine lymphocytes. II. Nature of the responding cells. J. Exp. Med. 145:163–174; 1977.
3. Suzuki, I.; Kumazawa, Y.; Miyazaki, T.; Mizunoe, K. Modulation of the antibody response to sheep erythrocytes in murine spleen cell cultures by a T cell mitogen extracted from *Bordetella pertussis*. Microbiol. Immunol. 22:47–51; 1978.
4. Cunningham, A. J.; Szenberg, A. Further improvements in the plaque technique for detecting single antibody-forming cells. Immunology 14:599–600; 1968.
5. Rich, R. R.; Pierce, C. W. Biological expression of lymphocyte activation. III. Suppression of plaque-forming responses in vitro by supernatant fluids from concanavalin A-activated spleen cell cultures. J. Immunol. 112:1360–1368; 1974.
6. Rodriguez, B. A.; Rich, R. R.; Rossen, R. D. Determinants of differential migration of mouse spleen cells treated with concanavalin A. J. Immunol. 115:771–776; 1975.
7. Machida, A.; Kumazawa, Y.; Mizunoe, K. Regulation of anti-hapten antibody response by chemically modified carrier antigen preferentially provoking delayed-type hypersensitivity. II. DTH-reactivity and carrier-specific suppression of anti-DNP antibody response induced by priming with dodecanoyl-BSA are mediated by functionally distinct T cell subpopulations. Microbiol. Immunol. 21:439–449; 1977.

Induction of High IgE Antibody Responses to Tetanus and Diphtheria Toxoids in Mice

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ABSTRACT

Barrier bred BALB/cStr Nctr female mice were primed by subcutaneous (SC) or intraperitoneal (IP) injections of DTP consisting of 2 LF diphtheria toxoid, 1.20 ou pertussis organisms and selected doses of tetanus toxoid (DTP) (Connaught, Inc.). The animals were challenged 28 or more days later with tetanus toxoid or with both tetanus and diphtheria toxoids. Serum samples were obtained at day 10 or 14 after challenge and biological assays were performed for tetanus antitoxin and for IgE antibody using a 48-hour heterologous passive cutaneous anaphylaxis (PCA) test.

Tetanus toxoid at dose levels of 0.004 LF and higher, administered with pertusssis vaccine, primed the mice for both tetanus antitoxin and for IgE antibody responses. A dose level of 0.4 LF tetanus toxoid was found to be optimal for tetanus priming. The pertussis vaccine was compared with other adjuvants for its capacity to augment tetanus antitoxin production. Although equivalent antitoxin titers were obtained when 5 mg aluminum hydroxide gel (Amphogel, Wyeth) was administered along with the tetanus, the secondary IgE titers were approximately tenfold greater. Evans Blue and Trypan Blue were also found to augment priming for high IgE antibody responses.

Pertussis vaccine did not augment the secondary tetanus or IgE antibody responses when administered at the time of challenge. When the challenging doses of tetanus toxoids and/or diphtheria were adsorbed onto activated charcoal an augmentation of IgE antibody occurred. Further augmentation was obtained when the primed mice were treated 3 days prior to challenge with 100 to 200 mg/kg of cyclophosphamide, a known immunosuppressant.

The data indicate that DTP induces priming for IgE antibody as well as other classes of immunoglobulin. Under normal conditions, production of the IgG class of antibody predominates, but if the animals are exposed to immunosuppressants such as cyclophosphamide at a critical time, IgE production is greatly augmented and under certain conditions may even predominate.
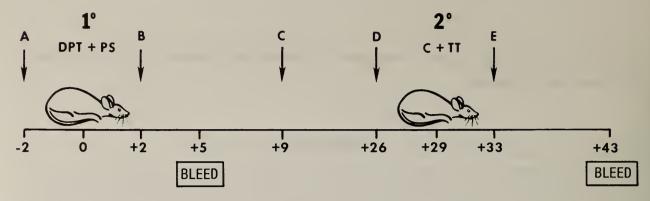
INTRODUCTION

There is a great need for procedures to screen potentially hazardous substances in our environment for their effect on immune mechanisms. At the National Center for Toxicological Research we are involved in designing models for assessing immunocompetence in mice whose immune mechanisms may be altered by exposure to toxic agents. Mice are exposed to potential toxicants and monitored for their capacity to respond to a battery of antigens. The antigens used are vaccines licensed for human use that have also been widely used in animal experiments. They consist of tetanus and diphtheria toxoids (T-dependent antigens) and pneumococcal polysaccharides (T-independent antigens) using pertussis vaccine as an adjuvant.

In the course of these experiments we noted that when tetanus toxoid was mixed with pertussis vaccine and injected subcutaneously, the animals were primed for high IgE and IgG production. However, under normal conditions, the IgE production is inhibited by the presence of regulatory cells induced at the time of priming. These regulatory cells are susceptible to specific drugs, x-irradiation and a variety of biological agents. When the regulatory cells are perturbed by a drug such as cyclophosphamide, an augmentation and persistence of the IgE response is observed on reexposure to tetanus toxoid.

MATERIALS AND METHODS

The experimental protocol we use to determine effects of drugs and toxic agents on the immune response is outlined in Figure 1. Specific pathogen-free BALB/c female mice are primed subcutaneously (s.c.) with an antigenic mix consisting of 0.4 Lf tetanus toxoid, 2 Lf diphtheria toxoid, 1.28 ou pertussis vaccine (Connaught, Inc.) and 0.5 μg

Figure 1. **Experimental Design.** Proposed model for detecting agents that inadvertently modify immune responses.

of either a single (Type SIII) or a polyvalent pneumococcal vaccine (Merck, Sharp and Dohme). Twenty-nine or more days later they are reinjected intraperitoneally (i.p.) with 1 Lf tetanus toxoid adsorbed on carbon or mixed with aluminum hydroxide gel. The drugs or toxic agents to be tested are given as single or multiple injections at selected intervals indicated by the arrows (A, B, C, D, E) in Figure 1.

Blood samples taken at selected intervals are assayed for IgM antibodies to the polysaccharide antigens, IgG neutralizing antibody to tetanus toxin, and IgE antibody to tetanus toxoid. The techniques have been described earlier (1). Briefly they consist of a radioimmunoassay method developed by Schiffman (2) in which nanogram quantities of S–3 antibody nitrogen per ml (ng Ab N/ml) can be determined in test sera by reference to a standard curve. Tetanus antitoxin titers are determined by a modification of a bioassay of Ispen (3), based upon the ability of serial dilution of serum to neutralize standard amounts of tetanus toxin when the mixture is injected intraperitoneally into 5–8 week old mice. IgE antibody to tetanus toxoid is measured by the standard passive cutaneous anaphylaxis assay (PCA) (4). Briefly, dilutions of mouse serum in physiologic saline containing 0.1% gelatin are injected intradermally (i.d.) in the rat and 1–3 days later 1 ml of 1% Evans Blue containing 100 Lf of tetanus toxoid is injected intrave-

nously (i.v.). The reciprocal of highest dilution of serum which results in a circular bluing of the skin over an area of 5 × 5 mm or more is recorded as the IgE titer.

RESULTS

A primary immunizing dose of 0.4 Lf of tetanus toxoid resulted in relatively high IgG antitoxin titers (equivalent to 90,000 neutralizing units per ml) and low IgE antibody titers (approximately 400 PCA units per ml). When mice were also exposed to an immunosuppressant such as cyclophosphamide at selected intervals before or after priming, a predictable suppression of IgM and IgG antibody titers was recorded (Table 1). However, the IgE titers in

Table 1. **Effect of Cyclophosphamide Administered Two Days After Priming on the Antibody Responses to Challenge with Tetanus Toxoid in BALB/c Female Mice**

Number of Mice	Dose of Cyclophosphamide i.p.	Antibody Titers S3 (ng Ab N/ml) (IgM)	Tetanus Antitoxin (IgG)	PCA Titers (IgE)
30	0	925	90,000	400
6	50	88	90,000	1,600
6	100	49	44,000	1,600
6	200	58	10,500	800

1° 0.4 Lf tetanus toxoid, 2 Lf diphtheria toxoid, 1.28 ou pertussis vaccine and 0.25 μg S3 in polyvalent vaccine sc.

2° Lf tetanus toxoid, 2 mg Al(OH)₃, and 0.25 μg S2 polysaccharide.

Animals were bled on day 43 (14 days after secondary injection).

these animals were greatly augmented; thus there appeared to be a dichotomy in the action of cyclophosphamide.

Specific priming for IgE antibody involves B cells bearing surface epsilon heavy chain determinants (B_e), which are regulated by both specific and nonspecific T-cell factors (5–7). Priming is an antigen-driven event easily modified by drug or chemical treatment. It was therefore of interest to determine the effect of various adjuvants on priming. Figure 2 illustrates the IgE and IgG antitoxin titers in mice primed with an antigenic mix consisting of 0.4 Lf of tetanus toxoid and various chemical agents. While all injections resulted in priming for both IgE and IgG antibody production, there was wide variation in the level of IgE antibody produced, depending upon the nature and dose of adjuvant used. Neither of the two types

of pertussis vaccine utilized (Connaught, Inc. Lots #1267–2 and #219) increased the PCA titers significantly over control levels, but at higher concentrations both appeared to suppress IgG antibody formation. When aluminum hydroxide gel at a dose level of 5 mg was used as the adjuvant, a marked elevation in IgE titers was obtained. Evans Blue at a dose level of 0.1 mg had a similar effect. Trypan Blue at doses of 3 and 6 mg inhibited priming for both IgG and IgE, but lower doses augmented priming for IgE. These results suggest that pertussis vaccine has minimal adjuvant effect on priming for IgE. However, this is not the case, as evidenced by the results obtained in a later experiment.

In the next experiment we compared the priming effect of pertussis vaccine and aluminum hydroxide gel, given s.c. or i.p., with and without cyclophos-

Priming		2°	IgE Antibody (PCA Units)	Antitoxin Titer per ml × 10^{-3}
T. Toxoid Dose Lf	Adjuvant	T. Toxoid Dose Lf	64 256 512 1024 2048	2 5 11 22 44 90 180 360
0.4	None (Saline)	1	▨ (to 256)	▨ (to 360)
0.4	4 OU HK PV	1	▨ (to 512)	▨ (to 44)
	1 " "	1	▨ (to 512)	▨ (to 44)
	0.25 " "	1	▨ (to 512)	▨ (to 90)
0.4	32 OU PVC	1	▨ (to 512)	▨ (to 44)
	8 " "	1	▨ (to 256)	▨ (to 44)
	2.7 " "	1	▨ (to 256)	▨ (to 44)
	0.9 " "	1	▨ (to 256)	▨ (to 44)
0.4	15 mg Al(OH)$_3$	1	▨ (to ~150)	▨ (to 90)
	5 " "	1	▨ (to 2048)	▨ (to 90)
	1.7 " "	1	▨ (to 1024)	▨ (to 44)
	0.6 " "	1	▨ (to 512)	▨ (to 44)
0.4	1 mg Evans Blue	1	▨ (to 512)	▨ (to 44)
	0.3 " "	1	▨ (to 512)	▨ (to 44)
	0.1 " "	1	▨ (to 2048)	▨ (to 90)
0.4	6 mg Trypan Blue	1	▨ (to 64)	▨ (to 5)
	3 " "	1	▨ (to 64)	▨ (to 22)
	1 " "	1	▨ (to 1024)	▨ (to 90)
	0.3 " "	1	▨ (to 1024)	▨ (to 90)
	0.1 " "	1	▨ (to 1024)	▨ (to 90)

HK PV = Heat-killed Pertussis Vaccine (Connaught)
PVC = Pertussis Vaccine Concentrate (Connaught)

· 101-77-44

Figure 2. Effect of adjuvant on priming for IgE and antitoxin responses. 14-day titers following challenge. Pooled data at 5 mice/group.

phamide treatment either s.c. or i.p. The cyclophosphamide treatment was given 3 days before reinjection of tetanus toxoid. This period was selected since it is known to reduce the effective T-suppressor cell population (8,9). A previous experiment had shown that the aluminum hydroxide induces relatively high IgE titers when given i.p. (Table 1). Cyclophosphamide treatment, however, enhanced IgE titers with either i.p. or s.c. injections (Fig. 3). When the priming adjuvant was pertussis vaccine given s.c. and cyclophosphamide was administered 3 days before secondary injection by the i.p. route, very high IgE titers were obtained. This represented a twelvefold increase over animals treated in the same manner but not injected with cyclophosphamide. This high response indicated that priming for IgE must have occurred following the s.c. injection of tetanus toxoid plus pertussis vaccine, but regulatory cells capable of suppressing IgE production were also produced. When the regulatory cells are perturbed by the drug treatment, suppressive action is inhibited and the B_e cells readily convert to antibody producing cells.

DISCUSSION

Pertussis vaccine, an adjuvant that induces a wide variety of hematological and immunological changes, has been widely used since 1947 (10). Kind (11) reported that pertussis vaccine sensitizes mice for anaphylaxis, and since then several workers have reported that it preferentially enhances IgE production (for reviews see refs. 12–14). Hardegree, et al. (in press, 1978) mixed tetanus toxoid with various adjuvants and noted that pertussis vaccine characteristically induced elevated IgE antibody titers 3 weeks after priming. Our experiments indicate that a further increase in IgE titers occurs in animals primed with tetanus toxoid plus pertussis vaccine and reinjected with the toxoid 28 days later. We also observed that cyclophosphamide applied at selected periods prior to secondary injection induced further enhancement of IgE titers. The action of the drug was directed at IgE induction and was obtained in animals primed by s.c. injection and challenged by the i.p. route.

Pertussis vaccine apparently can act as a nonspecific proliferating stimulus, recruiting cells that

Figure 3. Effect of route of priming and cyclophosphamide injection on the induction of IgE antibodies and tetanus antitoxin.

CP - Cyclophosphamide 200 mg/Kg

78-23
BALB/c ♀

subsequently become involved in antibody production (15). Suko et al. (16) reported an increase in the number of T-helper cells capable of acting on B cells programmed for IgE production. Both T-helper and T-suppressor cells are known to be involved in immunological memory (17,18), and suppressor cells or their precursors have been shown to be highly sensitive to cyclophosphamide (8,9). Our studies suggest that those T-helper and T-suppressor cells involved in the production of IgE are markedly augmented by pertussis vaccine and that treatment with cyclophosphamide inhibits T-suppressor cell activity, thereby permitting the T-helper cells to express themselves more completely. Thus the action of the drug is manifested by an alteration or shift in the immune response leading to high IgE titers that are potentially detrimental to the host. Similar regulatory disturbances resulting in elevated IgE titers are reported in patients with defects in the T-cell system, such as DiGeorge syndrome, Wiskott Aldrich syndrome, and thymic alymphoplasia (12,13).

The adverse allergic reactions reported in some children and adults repeatedly injected with DTP (19,20) may be due to 1) toxic factors in the vaccine or in the culture media used to cultivate the microorganisms used in preparing the vaccine; 2) genetic predisposition to react adversely to certain components of the vaccine; and 3) drugs, toxic agents, and environmental situations that modulate the immune response to cause sensitization for local or systemic reactions after later exposures to the vaccine components. Some of the chemicals and agents that augment IgE production are already known (endotoxin, x-irradiation, cholera toxin, parasitic infections, and antilymphocyte serum). It seems plausible that urban stress, a broad term used by Dr. Gordon Stewart to include the general conditions in highly deprived neighborhoods, may also predispose the individual to high IgE type reactions.

REFERENCES

1. Speirs, R.; Benson, R. W.; Schiffman, G. Models for assessing the effect of toxicants on immunocompetence in mice. Part 1: The effect of diphtheria, pertussis and tetanus vaccine on antibody responses to type III pneumococcal polysaccharide. J. Environ. Pathol. Toxicol. 1:689–699; 1978.
2. Schiffman, G.; Austrian, R. A radioimmunoassay for the measurement of pneumococcal capsular antigens and of antibodies thereto. Fed. Proc. 30:658 (Abst.); 1971.
3. Ipsen, J. Bioassay of 4 tetanus toxoids (aluminum precipitated) in mice, guinea pigs and humans. J. Immunol. 70:426; 1953.
4. Mota, I.; Wong, D. Homologous and heterologous passive cutaneous anaphylactic activity of mouse antisera during the course of immunization. Life Sci. 8:813; 1969.
5. Okumura, K.; Tada, T. Regulation of homocytotropic antibody formation in the rat. VI. Inhibitory effect of thymocytes on the homocytotropic antibody response. J. Immunol. 107:1682–1689; 1971.
6. Kishimoto, T.; Ishizaka, K. Regulation of antibody response in vitro. V. Effect of carrier-specific helper cells on generation of hapten-specific memory cells of different immunoglobulin classes. J. Immunol. 111:1–9; 1973.
7. Chiorazzi, N.; Fox, D. A.; Katz, D. H. Hapten-specific IgE antibody responses in mice. VII. Conversion of IgE "non-responder" strains to IgE "responders" by elimination of suppressor T cell activity. J. Immunol. 118: 48–54; 1977.
8. Polak, L.; Turk, J. L. Reversal of immunological tolerance by cyclophosphamide through inhibition of suppressor cell activity. Nature 249:654–656; 1974.
9. Ferguson, R. M.; Simmons, R. L. Differential cyclophosphamide sensitivity of suppressor and cytotoxic cell precursors. Transplantation 25:36–38; 1978.
10. Greenberg, L.; Fleming, D. S. Increased efficiency of diphtheria toxoid when combined with pertussis vaccine. A preliminary note. Can. J. Public Health 38:279; 1947.
11. Kind, L. S. Relationship of the anaphylaxis sensitizing and adjuvant properties of Hemophilus pertussis vaccine. J. Immunol. 79:238–242; 1957.
12. Tada, T.; Okumura, K.; Taniguchi, M. Cellular basis of IgE antibody formation in the rat. Ishizaka, K.; Dayton, D. H., Jr., eds. The biological role of the immunoglobulin E system. Washington, D. C.: U.S. Government Printing Office; 1972:89–102.
13. Taniguchi, M. The site of action of immunosuppressive agents in the primary antibody response of the rat with special reference to IgE antibody formation. Acta Pathol. Jpn. 24:449–464; 1974.
14. Clausen, D. R.; Munoz, J.; Bergman, R. K. Reaginic type of antibody in mice stimulated by extracts of Bordetella pertussis. J. Immunol. 103:768; 1969.
15. Finger, H. Possible mechanism of adjuvant action. Int. Arch. Allergy 36:293; 1969.
16. Suko, M.; Ogita, T.; Okudaira, H.; Horiuchi, Y. Preferential enhancement of IgE antibody formation by Bordetella pertussis. Int. Arch. Allergy Appl. Immunol. 54:329–337; 1977.
17. Miller, J. F. A. P.; Sprent, J. Cell-to-cell interaction in the immune response. VI. Contribution of thymus-derived cells and antibody-forming cell precursors to immunological memory. J. Exp. Med. 134:66–82; 1971.
18. Loblay, R. H.; Pritchard-Briscoe, H.; Basten, A. Suppressor T-cell memory. Nature 272:620–622; 1978.
19. Facktor, M. A.; Bernstein, R. A.; Fireman, P. Hypersensitivity to tetanus toxoid. J. Allergy Clin. Immunol. 52:1; 1973.
20. White, W. G.; Barnes, G. M.; Barker, E.; Gall, D.; Knight, P.; Griffith, A. H.; Morris-Owen, R. M.; Smith, J. W. G. Reactions to tetanus toxoid. J. Hyg. (Camb.) 71:283; 1973.

DISCUSSION OF PART 6

DR. HEWLETT: Dr. MacLennan, in the legend below the picture you showed of glutamine synthetase, there was mention of cyclic AMP binding to the enzyme. I wonder whether you or Dr. Zakharova can say whether there is any indication that cyclic AMP influences the activity of the glutamine synthetase in this organism?

DR. MACLENNAN: I have no information on that point. It was an illustration taken from a text.

DR. MUNOZ: Dr. MacLennan, when your preparation of the HA, the hemagglutinin in the fimbriae, is given in large doses, does it sensitize mice to histamine, or does it cause lymphocytosis?

DR. MACLENNAN: We have not really studied the homogeneity of the fimbrial hemagglutinin in preparations that we have made. They are certainly heterogeneous. They certainly contain two or three different antigenic components. If you raise an antiserum against the fimbrial hemagglutinin incorporated in Freund's Complete Adjuvant and examine antigenic heterogeneity by immunodiffusion, then you have three lines of precipitation. The most prominent of these lines is an antigen common to all *Bordetella* species. We are puzzled by this, and I think I suggested in my abstract that the fimbrial hemagglutinin is common to all *Bordetella* species, but we now believe that the common antigen is not apparently the fimbrial hemagglutinin line.

If you put high concentrations of fimbrial hemagglutinin, as much as 500 μg/ml, on immunodiffusion plates, you get a second, weaker line nearer the antigen reservoir, and the ability to form that line is removed by adsorbing the antigen preparation with red cell stroma. We believe that more likely represents the fimbrial hemagglutinin.

Fimbrial hemagglutinin appears to be present in some strains of *Bordetella bronchiseptica* when isolated from natural infections in marmosets. We have had two strains of *B. bronchiseptica* which produced fimbrial-type hemagglutination in 2 or 3-day-old liquid cultures. The hemagglutination is cholesterol-inhibited. If you immunize rabbits with such a strain of *B. bronchiseptica* but not with other strains of *B. bronchiseptica,* you get an antiserum which is inhibitory for *B. pertussis* fimbrial hemagglutinin.

We can measure the titer of antifimbrial hemagglutinin, as we can measure the titer of anti-LPF, by an inhibition of hemagglutination tests. I think this is quite interesting in light of our rabbit infection model, which I did not have a chance to describe. If rabbits have previously had *B. bronchiseptica* infection, we are unable to infect them with aerosols of *B. pertussis*. But if they have not had this infection, as shown by nasal swabbing and by serum antilipopolysaccharide estimations, we can infect them by inhalation of an aerosol of *B. pertussis* at 10^5 organisms per liter for 5 minutes. The nasopharynx is colonized for periods up to 50 days. Could *B. bronchiseptica* infection protect against subsequent *B. pertussis* infection by immunization against fimbrial hemagglutinin?

DR. GOTSCHLICH: Dr. MacLennan, what is the cholesterol concentration necessary in the liposomes to effectively adsorb the hemagglutinin? There is an ulterior motive for asking that question. If the hemagglutinin attaches to cholesterol, why is there specificity for cilia—unless, of course, cilia obtained an extraordinary amount of cholesterol on their membranes? Perhaps then you can see some particular inflection point in cholesterol concentration in the liposomes where the specificity exhibits itself.

DR. MACLENNAN: The molar ratio of egg yolk lecithin, dicetylphosphate, and sterol was 5:1:5 in the liposome preparations.

I wondered when I saw Dr. Sato's adhesion of cells to nonciliated cells and cells in culture, whether that might not be the fimbrial type of adhesion to a sterol, whereas perhaps the adhesion to the cilia in the respiratory tract might represent the LPF strong-binding activity to sialoprotein.

DR. RELYVELD: Data on the development of tetanus and diphtheria IgE after boostering with toxoids adsorbed onto aluminum compounds have been recently reported in the literature. The increase in specific IgE was not statistically significant for tetanus, but was for diphtheria in either atopic or nonatopic subjects. The ability of aluminum phosphate to stimulate anaphylactic antibodies to tetanus was reported in the last issue of *Allergy*. It was found that boostering with aluminum phosphate but not with calcium phosphate adsorbed toxoid led to a prolonged synthesis of specific IgE, and it has been assumed that the periodic use of vaccines adsorbed onto aluminum compounds could be related to an increased frequency of allergic diseases.

DR. STEWART: I am interested in Dr. Speirs' suggestion that IgE is produced under experimental conditions. I wonder if anyone had any evidence of what would happen in human subjects in this regard? From the reports that we have had through the Committee on the Safety of Medicines—and incidentally, may I say that the figures that I gave this morning were authorized by that Committee—we have had such cases in the United Kingdom. There are an appreciable number of children who have reacted with anaphylactic signs and this is also apparent in the American literature, especially the earlier American literature.

Now it is tempting to think that the very occasional severe reactions in vaccinated children may be related to the abnormal production of IgE because there are many other parallels with other drugs, for example, penicillin, where we know this can happen. And, at the same time, one wonders whether, in that event, if that child gets whooping cough, what kind of whooping cough does it get?

One of the things that first alerted me was the fact that we were getting some very severe cases of whooping cough in fully vaccinated children who had also had reactions to the vaccine. We have documented some of them. So it may be that, as Dr. Speirs says, there are two lines crossing here and I think that maybe I should say that when I speak of the environmental factors, I mean just that. I mean that there are other variables than just the vaccine and just *Bordetella pertussis*. There are other variables, but they are somewhat intangible and difficult to measure.

DR. SPEIRS: I do not think I have any thoughts other than what I tried to indicate, that there are a large number of factors that can modify or modulate the immune response. I was trying to point out some of those that should at least be taken into consideration as we do see these factors.

DR. MUNOZ: I wonder whether Dr. Speirs has any evidence to incriminate the macrophage as a suppressor cell.

DR. SPEIRS: I can't say that we have many years of experience with this particular problem. Our concepts have developed only recently in relation to these high IgE titers and the possibility that *B. pertussis* increases both the suppressor element as well as the helper elements. In other words, it increases the number of regulatory cells as well as the so-called B cells that can convert to IgE production, the B-Epsilon mark cells. I mentioned that Evans Blue and Trypan Blue act directly on macrophages and both lead to increased IgE production. I wonder whether the macrophage may not have a suppressive action on IgE responses. Anything that knocks out macrophages may increase IgE responses.

DR. KLEIN: I have a prepared statement on the differential effects of heat-stable and heat-labile components of pertussis on macrophage spreading.

I would like to stress the preliminary nature of these studies and point out that each data point represents the mean of four animals. The data, therefore, are not conclusive, but we feel they are certainly suggestive.

Despite the fact that *B. pertussis* is known to exert a profound and complex influence on immunological responsiveness, relatively little information exists on the cellular mechanisms involved. Recently, studies involving other microorganisms and their biologically active components have pointed to a relationship between altered macrophage function and altered immune responsiveness. With this in mind we have begun an examination of the influence of *B. pertussis* and its components on macrophage function. The studies reported here demonstrate that intraperitoneal injection of heated pertussis vaccine enhances the ability of explanted peritoneal macrophages to spread in vitro. However, injection of unheated vaccine fails to enhance spreading. These results suggest that heat-labile components of *B. pertussis* may suppress in vivo the effect of heat-stable components on macrophage function.

Methods. Pertussis vaccine was provided by Connaught Laboratories, Inc., Swiftwater, Pennsylvania. Organisms were grown on starch base/agar medium and preserved with thimerosal (1:5000). Histamine LD_{50} 5 days following vaccine injection was 1.8 mg base. Because *Cory-*

nebacterium parvum vaccine is known to alter macrophage function, it was also included in the present study. *C. parvum* vaccine was provided by Burroughs Wellcome Company as a formalin-killed suspension containing 1:10,000 thimerosal as preservative.

Groups of female inbred mice were injected intraperitoneally with 0.5 ml amounts of diluted vaccine containing 250 g (dry weight) of organisms. Peritoneal leukocytes were obtained by lavage and portions were used for total and differential leukocyte determinations, as well as for the spreading assay. The degree of macrophage spreading in vitro was determined according to the method of Bianco et al. Briefly, peritoneal cells from vaccine-injected animals were plated in culture medium in 35 mm petri dishes containing 13 mm glass coverslips. Cultures were incubated for 30 minutes at 37°C, rinsed three times with medium to remove nonadherent cells, and reincubated for 1 hour. Following incubation, coverslips were removed from culture, rinsed, and stained. The percentage of macrophages spread per coverslip was determined by scoring at 1000× magnification the number spread per 300 adherent mononuclear cells. Macrophages were considered spread when they displayed a membrane apron occupying an area double that of controls, or when they were elongated. A grid style micrometer eyepiece disc was used to aid comparison.

Results. Unheated pertussis vaccine tremendously augments the number of mononuclear cells in the peritoneal cavity. Heating the vaccine at 100°C for 30 minutes diminishes this effect. *C. parvum* does not significantly alter the mononuclear cell content of the peritoneal cavity.

Whereas Figure 1 demonstrated that pertussis vaccine significantly altered the number of peritoneal mononuclear cells, Figure 2 indicates that the spreading ability
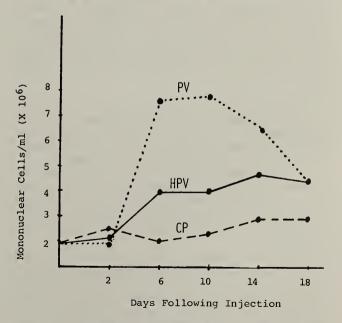
Figure 1. Peritoneal mononuclear cell response following intraperitoneal injection of *C. parvum* (CP), *B. pertussis* (PV), and heated PV (HPV).
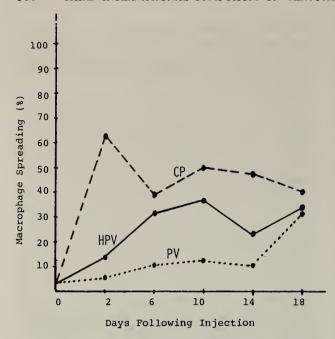
Figure 2. Spreading of peritoneal macrophages explanted from mice injected with *C. parvum* (CP), *B. pertussis* (PV), and heated PV (HPV).

of the cells is not significantly altered until late in the response. On the other hand, heated pertussis vaccine appears to induce a functional change in the cells leading to enhanced spreading ability. *C. parvum* has a marked effect on macrophage spreading.

Conclusion. These results demonstrate that *B. pertussis* can significantly alter macrophage function. In addition, they suggest that under certain experimental conditions heat-labile and heat stable components of *B. pertussis* may have differential effects on macrophage. In view of the central role of macrophage in immune responsiveness it will be of interest in future studies to examine the influence of more highly purified pertussis components on other parameters of macrophage function and define which components significantly alter macrophage function.

DR. ASKELÖF: I have a prepared statement on the effect of various methods for inactivating pertussis vaccines on agglutinogens, mouse protective activity, and the mouse weight gain test. This work was done in collaboration with L. Saarikoski, M. Tiru, and C.-R. Salenstedt.

The study was undertaken after we had found that heat inactivation of our pertussis vaccines quantitatively reduced or abolished their agglutinogen contents. On the other hand, vaccines inactivated with thimerosal without heating retained agglutinogens 1, 2, and 3 for more than 10 years.

In this short summary I will not go into any experimental details or discussion of results, but simply present the results obtained (a full paper is being prepared). The methods studied were heat inactivation at 56°C and inactivation with thimerosal at 4°C. Two different concentrations of thimerosal were investigated: 0.25%, which corresponds to 0.01 percent per 2×10^{10} bacteria in our final bulk; and a 0.01% final concentration. The thi-

merosal-inactivated vaccines were divided into two parts. One part was heated after various periods of inactivation with thimerosal, and the other part was analyzed without heating.

During the course of one year a total of more than 100 samples from four different vaccine preparations were analyzed according to the laboratory tests shown in Table 1.

Table I. Laboratory Tests

Mouse Protective Antigen
Agglutinogens
Lethal Toxin

Table 2 gives the effect of the different methods of inactivation on agglutinogens 2 and 3, evaluated by titration of sera from mice. The average titer for each inactivated vaccine over the course of 1 year was calculated. The three averages were then summed and each average titer expressed as a percent of the sum. The higher the percentage, the higher the titer. It is seen seen that inactivation with thimerosal for a period of up to 1 year and then heating is preferable to the other methods investigated.

Table 2. The Effect of Various Inactivation Methods on Agglutinogens

Inactivation Method	Aggl. 2	Aggl. 3
Heat	16 ± 13	21 ± 15
Thimerosal	22 ± 17	31 ± 35
Thimerosal + Heat	62 ± 23	48 ± 37
Σ %	100	100

Conclusion: Thimerosal + heat is preferable.

Table 3 shows the probabilities that the observed differences in the mouse protective activity of vaccines prepared by the different methods of inactivation occurred by chance. For example, inactivation with thimerosal without heating is better than heat inactivation ($p < 0.001$), and is also better than heating the vaccine after inactivation with thimerosal ($0.01 < p < 0.02$), which in turn is also better than heat inactivation ($0.01 < p < 0.02$). Finally, a high concentration of thimerosal (0.25% in this case) is superior to a low concentration (0.01%) ($0.001 < p < 0.005$), and is, in fact, superior to all the other methods tested.

Table 3. The Effect of Various Inactivation Methods on MPA

Thimerosal/Heat	$p < 0.001$
Thimerosal/Thimerosal + Heat	$0.01 < p < 0.02$
Thimerosal + Heat/Heat	$0.01 < p < 0.02$
Thimerosal, 0.25%/Thimerosal, 0.01%	$0.001 < p < 0.005$

Conclusion: Inactivation with a high thimerosal concentration (0.25%) is superior.

Table 4 shows the effects on the mouse weight gain test. Heating of the vaccines always abolished toxicity. Inac-

tivation with thimerosal without heating did not detoxify the vaccines until after one year. However, both methods are effective.

Table 4. The Effect of Various Inactivation Methods on MWG

Heat, 56° C, 10'/No treatment	p<0.0005
Thimerosal, 0.01%, 1 year/thimerosal, 0.01%, 2 months	0.005<p<0.01
Thimerosal, 0.25%/thimerosal, 0.01%	p<0.0005
Conclusion: Both heat and thimerosal (1 year) are suitable.	

Table 5 summarizes the previous tables. It can be seen that none of the methods was optimal with respect to all of the tests performed.

Table 5. Summary Results

Param-eter	Heat	Thimerosal (low)	Thimerosal (high)	Thimer-osal + Heat
MWG	+	+ (1 year)	+ (1 year)	+
MPA	−	−	+	−
Aggl	−	−	−	+
Conclusions: ?				

Critique of the Meeting

J. B. Robbins

Rather than review each paper or session, I will try to give you my thoughts about what we have learned that is new and what we might do with that new knowledge in the future.

I was very stimulated by the meeting, and I congratulate the organizers, who were wise enough to see its need for review of the rapid advances in this and related areas over the past several years, and the participants, who have presented creative and painstaking work.

The many people who labored to create this conference are to be commended, especially Drs. Manclark and Hill, for their foresight and energy in proposing the conference to the senior administrative staff. I personally commend Mrs. Connor, who quickly organized my notes for the critique I am about to present.

This meeting represents a tremendous advance over the previous pertussis meetings in the amount of new knowledge presented about the organism and the disease. These advances are directly due to the efforts of workers with long-term interest in pertussis, a disease that many people have considered to be an intellectually dead issue. These workers have continued to seek new knowledge and understanding of the disease and the action of the vaccine. Their patience, foresight, and persistence are acknowledged.

Our advances are also directly due to our improved ability to study the structure and physiology of bacteria, especially Gram-negative bacteria. Characterization of attachment factors and outer membrane structures has been especially helpful. Such basic scientific knowledge has found immediate application to our understanding of pertussis. This new information has made us appreciate what can be done in applying basic science to develop our knowledge of disease and its prevention.

The pertussis problem has captured the interest of the senior health officials of the United States and Canada. We assure them that their support of pertussis programs is not misplaced.

What have we learned here? We need to monitor disease caused by *Bordetella pertussis* and similar organisms that might mimic pertussis disease. We also need to monitor vaccine usage. However our surveillance techniques and coverage for both these problems are inadequate.

Our methods for detecting the organism and the disease have severe limitations. Dr. Broome's review of the fluorescent antibody reagents currently in use indicates their deficiencies. In addition, surveillance for pertussis must also include adults, and the whole problem of asymptomatic infection should be reviewed.

If pertussis is an exclusive infection of humans, it might be possible to eradicate it some day, just as we seem to have done with smallpox. I do not think the possibility is remote.

We have gained more intimate knowledge of the organism, especially the relation of its structure, function, and enzyme content of the phase variants that can be isolated and stabilized. But this raises questions. In studying the complexity of outer membrane structures in these phase variants, have we overlooked antigenic polymorphisms among the individual structures themselves? Can we use these high resolution techniques and genetic variants to study the structure, morphologic and function relations for individual components of pertussis?

We now have a molecular vision of the infectious process that allows us to visualize the initial interaction of the host and the parasite, and we can characterize that interaction by morphological and biochemical criteria. We see what happens to the activities of cells when

B. pertussis attaches to their cilia, and we now have an idea that alveolar macrophages may be the immune-induced effector cells in the lung parenchyma.

The successful efforts by the physical biochemists to isolate the components of the pertussis organism, such as the fimbriae and the whole host of biologically active substances, are commendable. I found it very exciting to see pictures of single structures that have now been assigned a function. Especially exciting were the isolated products of *B. pertussis* that exert a metabolic effect such as abnormal carbohydrate metabolism. The different susceptibilities of mice and rats to these products raises the possibility that individual variation among humans could explain why a few react abnormally to the vaccine. These products demand further study to determine their role in disease pathogenesis, vaccine toxicity, and disease-induced immunity.

It is interesting to consider that if one removed the word "pertussis" from our discussions, this forum would sound like one concerned with cholera toxin or endocrinology. This is because our discussions have so strongly emphasized the general principles of host-parasite interaction in the infectious process.

Given our current knowledge, our discussions of vaccine control have been disappointing. Though effort has not been lacking, it has been almost impossible to accomplish accurate characterization of potency or toxicity with regard to vaccines. Quantitative clinical studies are under way in the United States to assess vaccine reactions and to relate laboratory findings to the control testing of human reactivity of current and proposed vaccines. Dr. Manclark indicated how difficult it is, with current control procedures, to predict which vaccines are likely to cause more reactions than may be normally expected.

Unfortunately, we now know what happens when acceptance of pertussis vaccination declines: pertussis increases. The public's loss of confidence in vaccines in general is frightening, for it threatens a resurgence of other serious and preventable childhood diseases. We who are concerned with health issues have a great responsibility to base our arguments for vaccine usage on scientific methods; there is no room for distortion to bolster our views. Further vaccine programs should not be taken for granted; we should continuously examine their status quo.

What are the current barriers to our understanding clinical pertussis? We have difficulty defining patients to be surveyed. Our methods of detection are inadequate. Our techniques for culturing the organism are poorly standardized. Different laboratories should use standardized strains, grow them to a standard density, and plate them out on standard media so that clinical and quality control data from various laboratories can be comparable.

We have no serologic data to assess immunity. What will we measure? The current technique of using whole cells in the agglutination reaction is not a valid assay of specific immunity because of its high cross-reactivity. It cannot assay immunity, so we are unable to determine the level of herd immunity in a community. All we can do is wait to see what happens to the incidence of the disease as vaccine usage declines. Regulation of vaccine usage and evaluation of new vaccines would be helped greatly if we could accurately determine the level of immunity in individuals and in communities.

We now have some idea of the factors that govern the initial interaction between the pertussis organism and the host, but we do not know the critical host structures that are involved. Can we use our new understanding of other mucosal diseases? For example, it is now accepted that in cholera an attachment mechanism is required in addition to the metabolic effect exerted by the toxin. Attachment probably is an important pathogenic mechanism for diseases mediated by *Escherichia coli* and *Pseudomonas aeruginosa* as well. Unfortunately, we do not know the toxic component responsible for CNS injury in pertussis disease and which may occasionally occur after vaccination. Therefore we cannot accurately assess brain damage related to the use of pertussis vaccine.

Control of whole-cell vaccines, including those for pertussis, typhoid, cholera, and anthrax, is difficult. Standardization is difficult for all these vaccines, and there is no way to measure

the component that confers immunity in any of them. These problems are inherent in the use of whole cell vaccines, and it is clear to many workers that the best way to achieve protection with the least hazard is to isolate and characterize each component of pertussis vaccine.

What can we do with our existing information? I think we could start preparing serologic reagents for detecting purified components of the pertussis organism, using fluorescent antibody or other techniques, so that the potency and specificity of reagents can be characterized by different laboratories for comparison.

I am very enthusiastic about the initial observations on adverse reactions made by Dr. Hannik and her associates at the Rijks Instituut and the data on pertussis-induced alterations in carbohydrate metabolism generated by Dr. Ui and others. I feel that these two approaches might now be joined together in single studies. Altered carbohydrate metabolism is a likely mechanism for the adverse reaction to the vaccine. The study of altered carbohydrate metabolism might allow quantitative measurement of specific bacterial components and altered host response. I urge that such studies proceed as quickly as possible.

The pathogenesis of pertussis may now utilize individual components of the bacteria in immunologic assays and in vitro models. On the basis of the data presented to us, we could conceive an in vitro model using human cells to study antibody formation, the effect of bacterial components on individual respiratory cells, and the effect of adding the immune mechanism to the model. If bacterial components could be purified and used as vaccine components, this model system might allow their potency to be measured by physical and chemical methods. Perhaps the combination of the bacterial fimbriae (if they be the critical attachment factor) with their human cellular substrate could be analyzed by electron spin resonance. As we know from experience with meningococcal and pneumococcal vaccines, if the material that confers protective action can be characterized by physical and chemical techniques, the use of expensive and time-consuming bioassays can be avoided and the vaccine can be accurately controlled. A further advantage is that physical and chemical data from different laboratories can be more readily compared. Such techniques should be one of our goals as we work to develop new vaccines. However, it is implicit that the disease pathogenic steps and host immune mechanisms be identified.

The hypothesis for disease pathogenesis and immunity reviewed by Dr. Pittman is consistent with our current knowledge. It is a useful hypothesis because it predicts at least two disease- and vaccine-induced immune components. Therefore it may be critical to purify and characterize the structure and the morphological and functional relationships of several pertussis components.

There is a good possibility that the lymphocytosis promoting factor and other factors might be protective antigens. Studies should be done to see if their toxicity could be removed without destroying their immunogenicity. For various reasons, we have been restricted to inactivating pertussis cells with formalin, heat, or thimerosal, but there are new techniques that should be investigated, such as selectively altering the functions of these factors by attaching moieties to them under very mild conditions. With the availability of excellent methods for modifying proteins, we should not be restricted to existing techniques of reducing toxicity.

This conference has provided our political colleagues with a useful body of information for review and action. The point made by Dr. Cohen of the Rijks Instituut that more medically trained and experienced workers are needed to study vaccines and disease prevention is important. The cutback in research funds in the United States and many other countries has diminished this important human resource.

I want to conclude by reading the statement made by Dr. Mortimer in his review of vaccine usage. He said, "I would continue, and augment, current programs directed at widespread immunization by acknowledging that the vaccine does have problems, even though few. I would foster studies directed at defining precisely the biologic anatomy of the organism in

relation to man in an effort to determine what is immunogenic and what is toxic, and thus lead to a vaccine with a higher benefit-risk ratio."

I cannot add to that. It expresses exactly my opinions on the matter.

Again, I thank the participants for a very stimulating 3 days. I have one more suggestion—that there be no more pertussis conferences as such, and that the broader field of host-parasite interaction during the infectious process be the subject of our future meetings. At such meetings, students of pertussis would benefit by conferring with students of bacterial toxins, genetic factors in immune responsiveness, and bacterial physiology. In turn, the students of pertussis could provide the individuals in these other areas with information and inspiration.

Closing Remarks

V. J. Cabasso

As Dr. Robbins pointed out, many questions have been answered at this symposium, but many important ones remain unanswered. We have to know more about the safety and effectiveness of the vaccine. We have to know more about the pathogenesis of pertussis. And we have to know more about the action of the vaccines that have proved effective.

It is no wonder, then, that it took the combined muscle of the Bureau of Biologics, the National Institute of Allergy and Infectious Diseases, the Center for Disease Control, the Fogarty International Center, and the International Association of Biological Standardization to get us to roll up our sleeves, take stock of the situation, and pinpoint the areas that need more investigation.

This symposium has stimulated lively discussions and may have suggested new approaches to many of us. I am confident that the fruits of these deliberations will soon be evident.

I look forward to early publication of the proceedings of this conference so we can review and absorb this information more completely in the calmer atmosphere of our laboratories.

On behalf of our Association, I express satisfaction in having participated in the sponsorship of this important and productive symposium. We especially thank Dr. Manclark and Dr. Hill for their masterful organization of the symposium in every detail.

On behalf of all the sponsoring agencies, I thank the session officers and rapporteurs, the speakers and discussants, and all those in attendance for making this conference a real success.

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